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Immune response in dairy cow forestomachs

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Abbreviations

ACOD – Acyl coenzyme A oxidase

ACTH - Adrenocorticotropic Hormone

ALP – Alkaline Phosphatase

ANS - Autonomic Nervous System

APC – Antigen Presenting Cell

APPs – Acute Phase Proteins

ATF4 - Activating Transcription Factor 4

AVP - Arginine Vasopressin

AU – Arbitrary Units

BALT - Bronchus-Associated Lymphoid Tissue

BCS - Body Conditions Score

BHBA – β-hydroxybutyrate

bZIP - Basic Leucine Zipper

CALT - Conjunctiva-Associated Lymphoid Tissue

cDCs - Conventional Dendritic Cells

CLPs - Common Lymphoid Progenitors

CNS - Central Nervous System

CRH - Corticotrophin-Releasing Hormone

CRP – C-Reactive Protein

CV – Coefficient of variation

DAMPs – Damage-Associated Molecular Patterns

DC – Dendritic Cell

DHEA – Dehydroepiandrosterone

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

eIF2a - Eukaryotic Initiation Factor 2

ELISA – Enzyme-Linked ImmunoSorbent Assay

Fc – Constant region

FCS – Fetal Calf Serum

GALT – Gut-Associated Lymphoid Tissue

GGT – γ-Glutamyl Transpeptidase

GOT – Glutamic Oxaloacetic Transaminase

HDL – High-Density Lipoprotein

HEV – High Endothelial Venules

HMGB1 - High Mobility Group Box 1

Hp – Haptoglobin

HPA - Hypothalamic-Pituitary-Adrenal

IEC – Intestinal Epithelial Cells

IFN – Interferon

Ig – Immunoglobulin

IgA – Immunoglobulin A

IgD – Immunoglobulin D

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IL – Interleukin

IL1-R8 – Interleukin-1 Receptor 8

ILCs - Innate Lymphoid Cells

ILRs – Interleukin-1 Like Receptors

LALT – Larynx-Associated Lymphoid Tissue

LCA – Leukocyte Common Antigen

LPS – Lipopolysaccharide

LTi - Lymphoid Tissue-inducer

MALT – Mucosa-Associated Lymphoid Tissue

MBL – Mannose Binding Lectin

MHC – Major Histocompatibility Complex

MLN – Mesenteric Lymph Node

mTOR - Mechanistic Target of Rapamycin

NCR - Natural Cytotoxicity Receptor

NEB – Negative Energy Balance

NEFA – Non-Esterified Fatty Acids

NF-κB - Nuclear Factor κB

NK cell – Natural Killer cell

NLRs – NOD-Like receptors

NTC – No-Template Control

PAMPs – Pathogen-Associated Molecular Patterns

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction

PMNs – Polymorphonuclear leukocytes

POD – Peroxidase

PON – Paraoxonase

PP – Peyer's Patches

PRRs – Pattern Recognition Receptors

PTPRC – Protein Tyrosine Phosphatase Receptor type C

RNA – Ribonucleic acid

ROMs – Reactive Oxygen Metabolites

SAA - Serum Amyloid A

SARA – Sub-Acute Ruminal Acidosis

SCC – Somatic Cell Count

SH - Thiol groups

TAE – Tris-Acetate-EDTA

TBARS - Thiobarbituric Acid-Reactive Substances

Th – T helper

TLRs – Toll Like Receptors

TMR – Total Mixed Ration

TNF – Tumor Necrosis Factor

Treg – Regulatory T cell

VFA – Volatile Fatty Acid

Riassunto

Diverse patologie associate alla produzione colpiscono le vacche da latte e sono spesso considerate come indotte dall'uomo. Tali disordini si instaurano soprattutto durante il periodo del post-partum quando gli animali devono affrontare un bilancio energetico negativo (NEB), che i capi ad alto rendimento non sono in grado di bilanciare con una quantità energetica adeguata alle loro elevate esigenze produttive (Mulligan *et al.*, 2008).

Una corretta gestione delle malattie produttive richiede un approccio diagnostico precoce e dunque efficaci parametri prognostici. Nei ruminanti, lo stress metabolico influenza notevolmente la fisiologia dei prestomaci, inclusa la loro capacità di risposta immunitaria. Quindi una corretta valutazione delle malattie da produzione nei ruminanti dovrebbe anche comprendere markers della risposta immunitaria innata agli stress metabolici (Amadori, 2016).

Dal momento che la parete dei prestomaci esprime recettori immunitari e citochine e il liquido ruminale è infiltrato da leucociti, è ragionevole che essi svolgano un ruolo importante nella risposta immunitaria locale allo stress metabolico (Ingvartsen *et al*., 2003; Trevisi *et al*., 2014a).

La mia ipotesi di dottorato consiste nel fatto che il liquido ruminale possa essere un'importante fonte di informazioni diagnostiche, da affiancare alle analisi tradizionali per l'individuazione di allevamenti a rischio per le malattie da produzione.

A tale scopo, abbiamo caratterizzato le sottopopolazioni dei leucociti presenti nel liquido ruminale, rilevando la presenza di linfociti B, linfociti T e cellule mieloidi. In particolare i linfociti B si sono rivelati la popolazione leucocitaria più frequente nel liquido ruminale.

Abbiamo anche confrontato la composizione leucocitaria in campioni ottenuti mediante prelievo con sonda esofagea rispetto a quelli ottenuti con ruminocentesi, senza osservare alcuna differenza statisticamente significativa tra le due tecniche di raccolta.

Abbiamo investigato l'origine dei leucociti presenti nel liquido ruminale e siamo riusciti a dimostrare che essi derivano in parte dalla cavità orale e raggiungono il rumine attraverso la saliva.

Sulla base di questi risultati, abbiamo effettuato un’indagine sul campo di parametri dell'immunità innata nei liquidi ruminali di 128 animali provenienti da 12 aziende agricole, insieme ad una ispezione clinica, ad una valutazione dei parametri produttivi, alla misurazione del pH ruminale e alla presenza di acidi grassi volatili (AGV), e alla valutazione dei principali parametri metabolici ed ematologici. Quest'analisi di campo ci ha permesso di riscontrare correlazioni statisticamente significative tra marcatori immunitari e parametri biochimici. In particolare, è stata

riscontrata una correlazione negativa statisticamente significativa tra l'espressione genica del CD45 (marcatore dei leucociti) nel liquido ruminale e il pH ruminale. Inoltre abbiamo osservato che l'infiltrazione dei linfociti B è influenzata negativamente dal pH ruminale e dalle alte concentrazioni di acidi grassi volatili (AGV). Lo stesso tipo di regolazione è stata osservata nelle concentrazioni di IgM e Ig totali. È interessante notare che le Ig totali e le IgM nei liquidi ruminali mostrano una forte correlazione positiva con i livelli di urea nel sangue, in una certa misura correlati all'assunzione di sostanza secca da parte dell'animale.

Abbiamo poi indagato se una variazione della flora microbica ruminale potesse alterare il profilo infiammatorio del liquido ruminale, dimostrando che l'uso di una bassa dose di antibiotico (Monensin) ha determinato differenze significative nei parametri metabolici e immunologici nel rumine e ciò ha determinato una riduzione della colonizzazione dei leucociti e la concentrazione di immunoglobuline nel liquido ruminale.

Dalle osservazioni di campo è nata l'idea di sviluppare un kit per l'analisi del profilo infiammatorio del liquido ruminale, da affiancare alle più tradizionali tecniche diagnostiche per l'individuazione di malattie del peri-partum o disordini metabolici subclinici. Un prototipo di kit diagnostico è stato testato in un gruppo di 10 animali, divisi in due pool: pool A senza particolari problemi, pool B affetti da disturbi clinici (stasi ruminale). Le indagini molecolari, immunometaboliche e al FACS ci hanno permesso di individuare differenze tra i due gruppi. A livello molecolare il gruppo con patologia ha mostrato una marcata riduzione dell'espressione genica di IGLC e KRT5.

I nostri dati suggeriscono che la risposta immunitaria dei prestomaci potrebbe non solo essere diretta a "pericoli" che si manifestano localmente (ad esempio, sbilanciamento della dieta, fermentazioni anormali), ma potrebbe rappresentare un sistema reporter delle risposte di condizioni di malattia altrove nell'organismo.

Le aziende lattiero-casearie potrebbero essere classificate in base al loro profilo infiammatorio del rumine (popolazioni di leucociti o espressione di CD45) come a rischio o meno per le malattie da produzione. Le indagini diagnostiche sul profilo infiammatorio del liquido ruminale potrebbero integrare le metodiche diagnostiche tradizionali (come il pH del rumine o la conta delle cellule del latte) e contribuire a una diagnosi più precoce delle malattie produttive dei bovini da latte.

Abstract

Production diseases of dairy cows include several pathologies. These are considered man-made problems, mostly reported in the post-calving period of negative energy balance (NEB), when high-yielding dairy cows are unable to achieve a feed energy intake matching their high production requirements (Mulligan *et al.*, 2008).

A correct management of production diseases demands an early diagnostic approach and prognostic parameters. In ruminants, metabolic stress greatly influences the forestomachs physiology, including their immune response ability. Therefore a proper evaluation of production diseases in ruminants should also include markers of the innate immune response to metabolic stress (Amadori, 2016).

Since forestomach walls express immune receptors and cytokines, and the rumen liquor is infiltrated by leukocytes, it is reasonable that they play an important role in the local immune response to metabolic stress (Ingvartsen *et al.*, 2003; Trevisi *et al.*, 2014a).

My PhD hypothesis implies that ruminal fluids could be an important source of diagnostic information for the identification of herds at risk for production diseases, in addition to the traditional analyses.

Accordingly, we further characterized the leukocytes subpopulations in the rumen liquor, highlighting the presence of B cells (the most frequent leukocyte population in the rumen liquor), T cells, and myeloid cells.

We also compared the leukocyte composition in rumenocentesis versus esophageal probe samples, and we did not observe any significant statistical difference between the two sample collection techniques.

We investigated the origin of the leukocytes of the rumen fluid and demonstrated that they partly derive from the oral cavity and reach the rumen through the saliva.

On the basis of these findings, we carried out a field survey of innate immune parameters in rumen fluids of 128 animals from 12 farms, along with clinical inspections, assessment of milk yield, rumen pH and volatile fatty acids (VFA), and evaluation of major metabolic and hematologic parameters. Significant statistical correlations were found between immune markers in rumen fluids and biochemical parameters of dairy cows. In particular, a significant negative correlation was found between CD45 gene expression in rumen fluids (leukocyte infiltration) and ruminal pH. The infiltration of B cells was negatively affected by ruminal pH and high concentrations of volatile

fatty acids (VFA). The same type of regulation was also exerted on the concentrations of IgM and total Ig. Interestingly, total Ig and IgM in rumen fluids showed a strong positive correlation with urea levels in blood, to some extent correlated with feed intake.

We also investigated how the alteration of the ruminal microbiota could influence the immunologic profile of the rumen liquor. The use of a low-dose antibiotic (Monensin) led to significant differences in metabolic and immunological parameters in the rumen, and this resulted in a reduction of leukocyte colonization and immunoglobulin concentration in the ruminal fluid.

Our procedures were included into a kit and tested in a group of 10 animals, half of them suffering from ruminal stasis; we could detect differences between the two groups in terms of leukocytes infiltration and Ig concentration. At a molecular level, the group with overt pathology showed a marked reduction of IGLC and KRT5 gene expression.

Our data suggest that forestomach immune responses could be directed to “dangers” arising within the forestomach environment (diet unbalance, abnormal fermentations), but also arise as reporter system of disease conditions elsewhere in the body. The immune markers could integrate consolidated diagnostic parameters (e.g. rumen pH and VFA, milk cell counts, blood, fecal analytes) and contribute to robust, early diagnosis of production diseases of dairy cattle.

Introduction

Mammals have highly sophisticated digestive systems in order to take energy from a great variety of food types, and based on their types of digestive systems they can be divided in two main groups: monogastric and polygastric. Whereas most mammals fall in the category of monogastric, herbivores can be classified as either monogastric or polygastric (Sejrsen *et al.,* 2008).

Horses and rabbits are examples of monogastric herbivores; in these species, the fermentation chambers are represented by the cecum and the colon, both being well developed. On the other hand, in the polygastric herbivores the true glandular stomach is preceded by two or three pre-stomachs. On this basis, only polygastric herbivores can be classified as pseudo-ruminants and ruminants, respectively (Chilliard *et al.,* 2009; Millen *et al.,* 2016). Ruminants present three pre-stomachs and a true stomach, as shown in species like sheep, goats, deer, giraffes, antelopes, and cows (Sejrsen *et al.,* 2008; Millen *et al.,* 2016).

Bovine Forestomachs

The ruminant stomach is a big compartmentalized stomach that occupies 4/5 of the abdominal cavity, and consists of three no glandular compartments (rumen, reticulum, and omasum) lined with stratified squamous epithelium, and one compartment (abomasum) with glandular mucosa, that is also defined as the true stomach (Fig. 1).

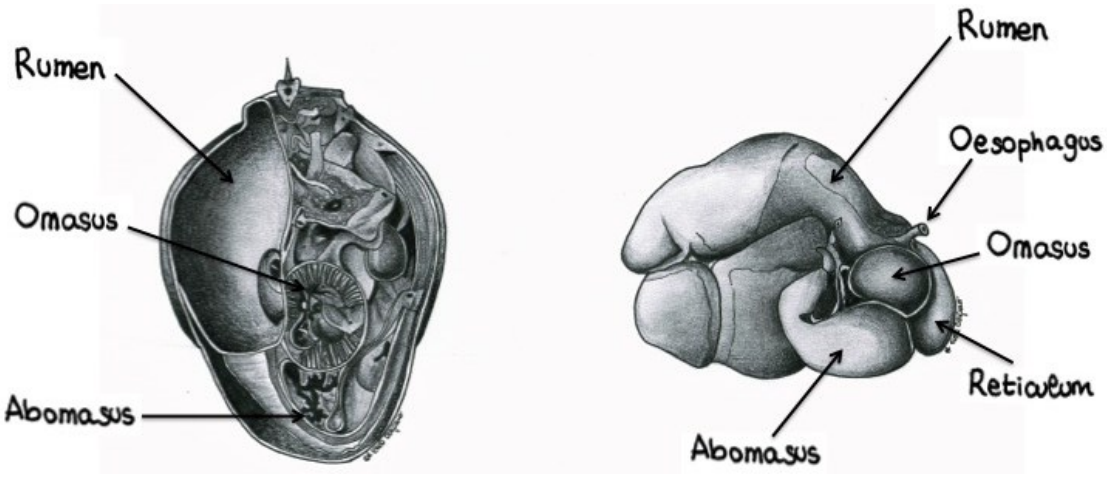


Figure 1 *– Left-section through cow's stomach, viewed from the front. Right - Cow's stomach viewed from the right-hand side. (Kindly drawn by Elena Deligant).*

The rumen is the first and the largest chamber of the ruminant stomachs, with most of the interior covered by papillae, and a medium capacity of 102–148 L. The surface of the rumen is

divided by right and left longitudinal grooves (thickened smooth muscle pillars) connected by cranial and caudal grooves into a dorsal sac and a ventral sac. Those rumen sacs communicate through the wide intraruminal orifice. The characteristics of the dorsal sac consist of a presence of a large gas bubble and its dorsal wall is free of papillae (Budras *et al.,* 2003; Frandson *et al.,* 2009).

Rumen mucosa (Fig. 2) is involved in different functions such as absorption, metabolism, and protection (working as a physical barrier, preventing the entry of microorganisms) (Lavker R. *et al.,*1969).

Figure 2 *- Rumen - detail of ruminal mucosa.*
(http://www.vivo.colostate.edu/hbooks/pathphys/digestion/herbivores/rumen_anat.html).

Under physiological conditions rumen manifests a continuous cyclical motility characterized by a series of contractions (that allows the mix of food for a more efficient digestion, the elimination of gases through eructation and the movement of food particles back to the mouth for a second mastication and rumination) (Chilliard *et al.,* 2009).

Rumination is one of the behavioral characteristics typical of ruminants and is constituted by a complex and coordinated stereotyped sequence of events. These stereotyped series of events are a complex reflex phenomenon and require an activation of peripheral sensory system. Rumination has also a circadian rhythm and is commonly associated with the state of drowsiness, its incidence being higher during the afternoon and in the middle of the night (Millen *et al.,* 2016).

The rumen is an ideal microbial habitat. Indeed, the internal part of the rumen maintains a relatively constant temperature (36–40°C), an anaerobic environment and a controlled pH (pH = 6-7) thanks to the buffering effect mainly provided by salivary secretions. The consumed water and the saliva (the only exocrine secretion that arrives into the rumen) provide a moist environment ideal for microbial growth (Millen *et al.,* 2016).

Under these conditions, the rumen is inhabited by a multitude of microorganisms, defined as “normal flora”, or as autochthonous microbiota. These microorganisms normally colonize and grow

in the rumen, living in a symbiotic relationship with the host that is absolutely dependent on them to digest fiber, since only those microorganisms have fibrolytic enzymes to degrade cellulose and hemicelluloses; they can also use nonprotein nitrogen (e.g. urea, nitrate, nucleic acids) as source of ammonia and synthesize amino acids and protein that can be used by the host. Additionally, the ruminal flora includes allochthonous microbiota, that are not established (colonization and growth) inside the stomach, i.e. they are latent and in transit. These microorganisms are mainly derived from ingested food and water or swallowed with the air, or even from other parts of the host (e.g. skin, respiratory tract, or reproductive tract). These nonindigenous microorganisms can also include a variety of pathogens that can actually colonize and grow to establish infections. Also, some members of the normal flora could assume pathogenic roles (opportunistic pathogens) when the ecosystem is perturbed in some way or when a breach occurs in the integrity of the ruminal wall (Chilliard *et al.,* 2009; Millen *et al.,* 2016).

The microbial population in the rumen includes members that belong to all three life domains, Eubacteria (Bacteria), Archaea (Methanogens), and Eukarya (Protozoa and Fungi), and can be divided into three groups – Bacteria, Protozoa, and Fungi (Chilliard *et al.,* 2009; Sejrsen *et al.,* 2008; Millen *et al.,* 2016). Rumen microorganisms can be also assigned to different functional groups, such as cellulolytics, amylolytics, proteolytics, etc., which degrade the wide variety of feed components or metabolize some of the products formed by other microorganisms (Hungate, 1966).

Therefore, the characterization of these communities is pivotal to understanding ruminal transformations of plant material to both undesirable and useful metabolic products (Henderson *et al.*, 2015). Without the microbiota, cellulose, hemicellulose and peptic substances, structural components of vegetables, would be otherwise impervious to hydrolysis by the alimentary canal gland secretions, none of which is capable of cleaving the β-glycosidic bond that characterizes the structure of these polysaccharides (Frandson *et al.,* 2009).

The bacterial pre-digestion allows the utilization of plant cellulose. Accordingly, forestomachs are used by ruminants as real proofers, where feed rich in cellulose first undergoes a degradation process carried out by an impressive variety and quantity of microorganisms (e.g. *Ruminococcus spp*, *Fibrobacter spp*). These microorganisms transform feed and lead to the liberation of volatile fatty acids (VFA); these are the main products of ruminal fermentation, and include acetic acid (± 65%), propionic acid (± 22%), and butyric acid (± 13%) (Chilliard *et al.,* 2009; Frandson *et al.,* 2009). The profile of VFA produced in the rumen has actually consequences on the host's metabolism. While propionic acid formation converts most energy in the rumen into useful

products, excessive propionic production can cause undesirable consequences on production, such as reduced intake (Oba *et al.*, 2003), and low milk fat content (Ørskov *et al.*, 1990).

The volatile fatty acids are then absorbed directly through the epithelium that covers the inside of the forestomach. The presence of the papillae (Fig. 2) guarantees an increased surface for absorption, necessary to dispose of all the gases and metabolites produced by the great number of microorganisms. In addition, microbial components are made available to the host such as microbial proteins of high biological value and easy to digest (Chilliard *et al.,* 2009; Frandson *et al.,* 2009).

The reticulum represents the second chamber of the ruminant stomach. In the digestion process, the reticulum mainly plays a mechanical role, sending the coarse parts to the rumen or the esophagus for a second chewing process, while the most elaborate part is sent towards the omasum.

Feeds that have undergone a second mastication, are richer in water and form a thick mush that falls directly into the reticulum, which conveys it to the omasum (Frandson *et al.,* 2009).

The omasum is the third chamber. The mucosa of the omasum forms large plicae, called laminae, which greatly increase its surface. The omasum has a keratinized stratified squamous epithelium, and this chamber does not contain any glands (Budras *et al.,* 2003; Chilliard *et al.*, 2009). Its wall is highly folded, thus providing a large surface area which allows for an efficient absorption of water and salts released from the partially digested food. As in rumen and reticulum, the absorption of VFA continues also in the omasum. Actually, the main function of this chamber is to regulate the transit of the feed from the reticulum to the true stomach, the abomasum, where acid digestion takes place (Chilliard *et al.,* 2009; Frandson *et al.,* 2009).

The abomasum presents a thin wall and is capable of great distension and displacement, with a capacity of up to 28 L. The functions of the abomasum are similar to those of the monogastric stomach, as regards the secretion of gastric juice (it secretes hydrochloric acid and pepsinogen). So its function is related to the chemical breakdown of feed. In the adult, this stomach compartment is pivotal to protein digestion, and such a task is particularly important in the calf before weaning: milk is piped directly into the abomasum, where it is rapidly coagulated and digested (Chilliard *et al.*, 2009; Frandson *et al.,* 2009).

Rumen microbiota and metabolism

It is well known that microbiota communities have a pivotal role in the host's health and welfare; possible changes in its composition or function have been demonstrated to have severe consequences on the host. This is especially true of the gastrointestinal tract of dairy cattle where microbiota can influence milk production and the host's health. Nevertheless, our understanding of bacterial communities in the gastrointestinal tract of dairy cattle is still very limited.

A study from 2015 of the mucosa-associated microbiota in the gastrointestinal tract of Holstein Friesian dairy cattle showed significant differences in bacterial prevalence and diversity between gastrointestinal regions. The main taxonomic groups represented within cattle gastrointestinal tract were Firmicutes, Bacteroidetes and Proteobacteria, but these varied considerably among regions both in abundance and in genera number, the forestomachs exhibiting greater relative abundances of Firmicutes and Bacteroidetes, whereas the intestine (except for the rectum) showed higher relative abundances of Firmicutes and Proteobacteria (Mao *et al*., 2015). These differences between gastrointestinal areas may be explained by the difference in the pH values, as well as by the gut motility, redox potential, nutrient supplies and the host's secretions (Salonen *et al*., 2014).

The dominant microbiota taxa identified in forestomachs belong to unclassified *Ruminococcaceae*, unclassified *Rikenellaceae*, unclassified *Christensenellaceae* and unclassified *Lachnospiraceae*, which have been detected mostly in the rumen, where it is thought that they play an important role in starch and fiber degradation (Kim *et al*., 2011).

The differences between digesta- and mucosa-associated bacteria and along the gastrointestinal tract in cattle are consistent with a pivotal role in feed digestion and metabolism of digesta-associated microbiota (in areas like the rumen), while the mucosa-adherent microbiota is involved in processes of epithelium proliferation and immune defense. These different functions performed by bacteria are essential for the host's survival and adaptation (Mao *et al*., 2015).

The rumen microorganisms ferment and degrade the plant fibers in a coordinated and complex manner which results in the conversion of plant materials into digestible compounds, such as volatile fatty acids and bacterial proteins. In ruminants, the predominant volatile fatty acid is acetate, which is produced by different bacteria present in dairy cattle forestomachs, like cellulose-degrading bacteria (e.g. *Fibrobacter succinogenes*, *Clostridium lochheadii*, and *Ruminococci albus*), amylolytic bacteria (e.g. *Bacteriodes ruminicola*, and *Ruminobacter amylophilus*), lipolytic bacteria (e.g. *Anaerovibrio lipolytica*), lactate-degrading bacteria (e.g. *Selenomonas lactilytica*, and *Megasphaera elsdenii*), pectin-degrating bacteria (e.g. *Lachnospira multiparus*) (Cotta, 1988; Weimer, 1996; Duskova *et al.*, 2001; Michalet-Doreau *et al*., 2002; Brown *et al.,* 2006; Fuentes *et*

al., 2009; Ivan *et al.,* 2012); while propionate is produced by amylolytic bacteria (e.g. *Selenomonas ruminantium*, and *Succinomonas amylolitica*), lipolitic bacteria (e.g. *Anaerovibrio lipolytica*), lactate-degrading bacteria (e.g. *Megasphaera elsdenii*) (Cotta, 1992; Brown *et al*., 2006; Fuentes *et al.,* 2009), and finally butyrate can be produced by Cellulose-degrating bacteria (e.g. *Butyrivibrio fibrisolvens*, and *Clostridium lochheadii*), and lactate-degrading bacteria (e.g. *Megasphaera elsdenii*) (Weimer, 1996, Brown *et al.*, 2006).

In order to direct the ruminal fermentations toward the production of more energetic VFA and a higher propionate:acetate ratio the use of probiotics, such as Monensin, is largely used in dairy cattle.

Monensin (Fig. 3) was originally isolated from a fungus (*Streptomyces cinnamonensis)* and used as antimicrobial compound for coccidiosis (0,5 mg/kg).

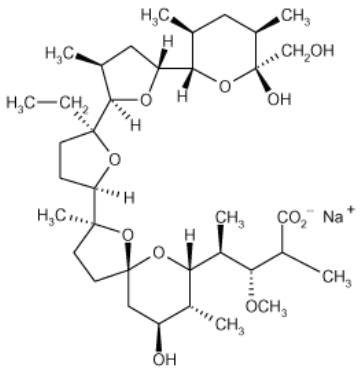


Figure 3 *– Monensin molecule ($C_{36}H_{61}O_{11}.Na$).*
(http://www.enzolifesciences.com/ALX-380-026/monensin-.-sodium-salt/).

Nowadays it is widely used in ruminants in order to promote the propionic acid production and prevent bloat, and also because of it anti-ketotic function (Łowicki *et al*. 2013; Butaye *et al*., 2003). It is an ionophore (ion conveyor) with a selected activity against $Gram^+$ rumen bacteria. It interacts with bacterial and protozoal membranes and facilitates monovalent cation/H^+ exchange, causing microbes to spend a lot of ATP to react to these changes until they eventually “run out". On the whole, it increases the production of the rumen propionic acid and fosters liver gluconeogenesis (Matsuoka *et al.*, 1996). Monensin promotes the reduction of $Gram^+$ bacteria (underlying fiber fermentation in the rumen). This way, $Gram^-$ bacteria (that favor starch fermentation) show an increased prevalence, and the volatile fatty-acid profile is changed. $Gram^-$ bacteria produce more propionate fatty acid, which is more energy dense than acetate, and a lower acetate:propionate ratio is a known benefit of feeding Monensin (Plaizier *et al*., 1997). If used before the peripartum,

Monensin can help prevent ruminal acidosis, and can also have effects on energy metabolism including reduced blood ketones, increased presence of gluconeogenesis precursors, and increased milk production (Duffield *et al*., 2000).

The use of Monensin in farm animals has always been described as safe and effective, but only when used at recommended dosages of up to 2 mg/kg (Potter *et al*., 1984; Duffield *et al.*, 2000), its toxicity for cattle being dose-dependent (Potter *et al.*, 1984, Hall *et al.*, 2000). The few cases of intoxication described in bibliography were a result of errors in the diet (Wentink *et al.*, 1981; Bastianello *et al.*, 1996; Hall *et al.*, 2000; Nebbia *et al.*, 2001). The Monensin LD_{50} for cattle has been estimated to be between 21.9 and 80 mg/kg (Potter *et al.*, 1984, Hall *et al.*, 2000; Nebbia *et al.*, 2001), so it is clear that is completely safe if used at the recommended daily doses (up to 2 mg/kg).

Given that the use of Monensin is a widespread practice due to its advantages on the rumen metabolism of dairy cows, we investigated how this molecule could modify the immunologic profile of the rumen liquor.

Immune system

The immune system of vertebrates, including cattle, is a dynamic, complex system that has as main goal the protection against pathogens (Gonzàlez *et al.*, 2012).

Immunity is actually defined as resistance to disease, specifically infectious diseases, and the immune system has evolved due to the constant pressure applied by the pathogens; it is responsible for recognizing, resisting, and eliminating those (Abbas *et al.*, 2016).

In order to fulfill its function, the immune system uses different types of physical barriers (e.g. skin and mucous membranes), cells (e.g. neutrophils, macrophages, natural killer cells, and lymphocytes), and humoral factors (e.g. complement, antibodies, and cytokines). Those different components interact with each other and work as a team. Following the interactions among immune cells, humoral factors and infectious agents, a series of chain reactions take place and together constitute the immune response (Halliwell *et al.*, 1989; Morrison *et al.*, 1986; Lippolis *et al.,* 2008; Poli *et al.*, 2017).

The defense mechanisms include physical barriers, the innate immune response that initiates the process, and the acquired immune response (which develops more slowly) that mediates a later and often more effective defense against infectious agents (Abbas *et al.,* 2016).

In the present introduction among the numerous aspects of the immune system I will focus only on some aspects directly pertaining to my thesis, i.e. mucosal immunity, the acute phase response, the effects of stress, and leukocytes traffic to the gastrointestinal tract.

Mucosal immune system

At mucosal surfaces the immune response is organized by the Mucosa-Associated Lymphoid Tissue (MALT), placed at strategical sites to allow for efficient antigen sampling from mucosal surfaces (Liebler-Tenorio *et al.*, 2006).

Once the antigens are ingested or inhaled, lymphocytes and antigen presenting cells (APCs) that colonize the mucosal surfaces are involved in the immune response. Mucosal epithelia are barriers between the external and internal environments and can be a site of entry of bacteria and other pathogens (Abbas *et al.*, 2016).

In MALT, the lymphoid tissue is in close contact with the mucosal surfaces and is structured as lymphoid follicles with T cell-dependent interfollicular areas (Liebler-Tenorio *et al.*, 2006).

The lymphoid follicles are mainly formed by B lymphocytes fixed in a network of follicular dendritic cells. In the follicles a small number of $CD4^+$ T lymphocytes and macrophages is also present, while in the interfollicular areas there is a predominance of $CD4^+$ and $CD8^+$ T lymphocytes (Liebler-Tenorio *et al.*, 2006).

The epithelium overlying the GALT is infiltrated by lymphocytes and may contain morphologically distinct cells specialized in the uptake of antigens. Those cells are named M cells based on their morphology in Peyer's patches (PP), where "M" is short for "membranous" cell. The primary physiological role of M cells seems to be the quick absorption of antigens and their presentation to the immune cells of the lymphoid follicles (Fig. 4), thereby inducing an effective immune response (Liebler-Tenorio *et al.*, 2006).

M cells acquire solid soluble substances (e.g. bacteria) by endocytosis via the apical membrane and by means of vesicles. The vesicles are released from the basolateral membrane by exocytosis and processed by macrophages, which present antigens to a T helper that will stimulate the production of IgA (Rojas *et al.*, 2002).

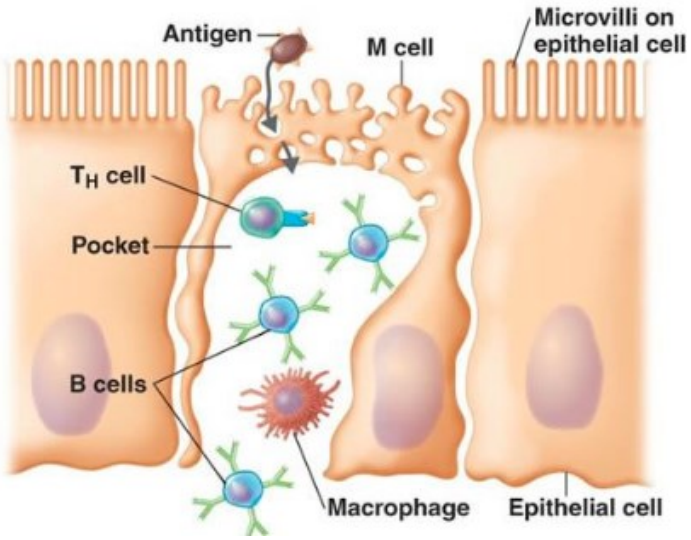


Figure 4 *– M cell – Facilitates the contact between antigens and leukocytes. (https://it.pinterest.com/pin/276267758369687527/).*

Recirculating lymphocytes enter MALT through the high endothelial venules (HEV) into the interfollicular areas. Distinct receptors expressed on HEV regulate the tissue-specific migration of lymphocytes (Liebler-Tenorio *et al.*, 2006).

Morphology, distribution, occurrence, ontogeny and evolution of MALT vary between species.

In cattle we can find:

- Conjunctiva-Associated Lymphoid Tissue (CALT),
- Lymphoid Tissues of Waldeyer's ring,
- Larynx-Associated Lymphoid Tissue (LALT),
- Bronchus-Associated Lymphoid Tissue (BALT),
- Gut-Associated Lymphoid Tissue (GALT).

Concerning the gastrointestinal tract the established knowledge on the mucosal immune system mainly refers to the intestinal region and very few data are available about the esophagus, stomach and ruminant forestomachs.

In the intestinal tract, MALT takes the name Gut-Associated Lymphoid Tissue or GALT. The activity of GALT aims to discriminate between pathogenic and commensal bacteria. The recognition of commensal microorganisms is essential for the development and function of the immune system in the mucosal and peripheral districts (Liebler *et al.*, 1988; Macpherson *et al.*, 2004).

Owing to its physiological function in food absorption, the mucosal surface of gut is thin and acts as a highly selective permeable barrier to entering the interior of the organism, thus carrying out a mechanical protective function. Equally, its fragility and permeability creates vulnerability to infection and, in fact, the vast majority of the infectious agents use this route (Murphy *et al.*, 2012). The functional importance of GALT in defending the organism relies, among other things, on its large population of plasma cells, whose number exceeds the combined number of plasma cells in spleen, lymph nodes and bone marrow (Kindt *et al.*, 2007).

The different lymphoid elements of GALT can be morphologically and functionally subdivided into two major structures: the organized GALT, consisting of mucosal follicles that are responsible for the induction phase of the immune response, and the diffuse GALT, which consists of widespread leukocytes scattered throughout the epithelium and the lamina propria of the mucosa. The organized GALT includes the ileal Peyer's patches (PP) and the mesenteric lymph nodes (MLN) (Murphy *et al.*, 2012).

The Peyer's patch is an aggregate of lymphoid cells facing the lumen of the gut which acts as a site of initiation of the immune response. It includes a subepithelial dome area where large number of B cell follicles with their germinal centers surrounded by a small number of T cells areas and dendritic cells are found. In this area, the subepithelial dome is separated from the intestinal lumen by a layer of follicle-associated epithelium. This contains conventional intestinal epithelial cells and a small number of specialized M cells. Unlike enterocytes, these M cells present a folded luminal surface instead of the microvilli, do not secrete digestive enzymes or mucus, and lack a thick surface of glycocalix. Those cells transport macromolecules from the intestinal lumen to the subepithelial tissue and are actively pinocytic (Aguilera Montilla *et al.*, 2004; Abbas *et al.,* 2016).

Diffused mucosa-associated lymphoid tissue includes the lamina propria mononuclear cells and the intraepithelial lymphocytes.

The intestinal lamina propria leukocytes comprise diverse cells: T lymphocytes (most of them are $CD4^+$ and have the phenotype of activated cells), B cells, macrophages, DCs, neutrophils, other granulocytes, and mast cells. The large number of macrophages, DCs and T cells in the lamina propria make it likely that antigens crossing the epithelium may be processed and presented to lamina propria $CD4^+$ T cells that have the important function of local immune regulation (Aguilera Montilla *et al.*, 2004; Abbas *et al.*, 2016).

Activated $CD8^+$ lymphocytes proliferate and, while some of them become memory cells, others differentiate into cytotoxic T lymphocytes capable of recognizing the antigen on the surface of infected cells, attacking them and causing their lysis and death (Castro-Sanchez *et al.*, 2013).

Intraepithelial lymphocytes represent a functionally heterogeneous population that contains cells with antitumor activity, natural killer activity, allospecific cytotoxic T lymphocytes, and precursors of cytotoxic T lymphocytes (Aguilera Montilla *et al.,* 2004). Among the cells present in the gastrointestinal mucosa we can also find innate lymphoid cells (ILCs).

ILCs have been described as a lymphocyte subfamily that have important roles in immune defense, inflammation and tissue renovation, and can be distinguished from other lymphocytes by their lack of clonotypic antigen receptors and by their developmental independence from DNA rearrangement (Liu *et al*., 2017a). Mature ILCs express the same surface receptors and effector molecules of differentiated T cells under the control of specific transcription factors, and present a lot of similarities with those cells. Morphologically, ILCs look like lymphoid cells and develop from common lymphoid progenitors (CLPs) that express CD127 (also known as IL-7Rα) (Björkström *et al.,* 2013). But maybe, the most interesting feature is their role as early effectors of immunity, being capable of responding within hours after activation, as opposed to other lymphocytes (Serafini *et al.,* 2015).

ILCs are normally located near commensal microorganisms in the gastrointestinal, respiratory and urogenital tracts and in the skin, and are susceptible to signals from epithelial and myeloid cells. Those signals seem pivotal to activating ILCs in case of infection but with potentially negative effects on microbial communities (dysbiosis); this means that the ILC response is clearly beneficial to the host, helping it to eradicate pathogens, but it could also be detrimental by affecting commensal flora (Guri-BenAri *et al*., 2016).

ILCs have been classified into three major groups (based mainly in phenotypic and functional resemblances to T helper (Th) cells), that are characterized by specific transcription factors and cytokine expression:

- ILC1 cells produce Th1-type cytokines (e.g. IFNγ) and include NK cells;
- ILC2 cells produce Th2-type cytokines (e.g. IL-5 and IL-13) and include natural helper cells, nuocytes and innate helper 2 cells;
- ILC3 cells include several phenotypically different subsets, including lymphoid tissue-inducer (LTi) cells and mucosal natural cytotoxicity receptor (NCR)-bearing cells that produce the Th17-type cytokines (e.g. IL-17A and IL-22) (Spits *et al*., 2013).

ILCs are often found at mucosal surfaces, where they can be exposed to infectious agents present in the environment.

The best known ILC1 are NK cells, and these cells are the major ILC population, with a potential anti-tumor role (Dadi *et al*., 2016).

ILC2 are a major early source of IL-13, which can activate T cells and induce physiological responses that will help against helminthic infection. These responses stimulate goblet cell mucus secretion and contraction of smooth muscle cells. In addition, they secrete signals that recruit and activate mast cells and eosinophils, thus stimulating B cell proliferation. They also secrete amphiregulin (member of the epidermal growth factor family) that can stimulate tissue repair (Palm *et al.,* 2012).

In the intestinal tract, ILC3 cells have a crucial role in mediating the balance between commensal microbiota and the intestinal immune system. In response to inflammatory signals from the dendritic cells and gut epithelium, they produce IL-22 which increases the production of antimicrobial peptides and defensins. ILC3 can also assist in immune responses to extracellular bacteria by maintaining epithelial homeostasis (Walker *et al*., 2013).

Recent single-cell genomic analysis of ILCs in the mouse small intestinal lamina propria showed that the commensal microbiota is required to maintain ILCs diversity. A study of the transcriptional and regulatory patterns of intestinal ILCs identified high levels of diversity under conditions of homeostasis that are maintained by the commensal microbiota and tend to an ILC3-like profile in the absence of microbial stimulation (Guri-BenAri *et al*., 2016). However, until this date no studies have been performed about the description of ILCs in cattle.

Coming back to the main role of the GALT, to discriminate between the commensal and pathogen microorganisms, much has been elucidated about the signal transduction pathways used by the innate immune system to fight invading microbial agents. It has been found that microbial components are recognized by intestinal epithelial cells (IEC) by means of pattern-recognition receptors (PRRs) that sense the presence of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).

In mammalian cells, LPS and other bacterial molecules are recognized by a class of pattern-recognition receptors PRRs known as Toll-like receptors (TLRs) (Fig. 5), and NOD-like receptors (NLRs), able to signal and initiate the immune response. The activation of TLRs and NLRs results in the expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides, initiating and regulating the immune response. Regulated expression (down-regulation under steady-state conditions) and localization (intracellular or basolateral localization) of TLRs and NLRs are proposed as possible mechanisms limiting the recognition of commensal bacteria (Abreu *et al.*, 2001; Uehara *et al.,* 2007).

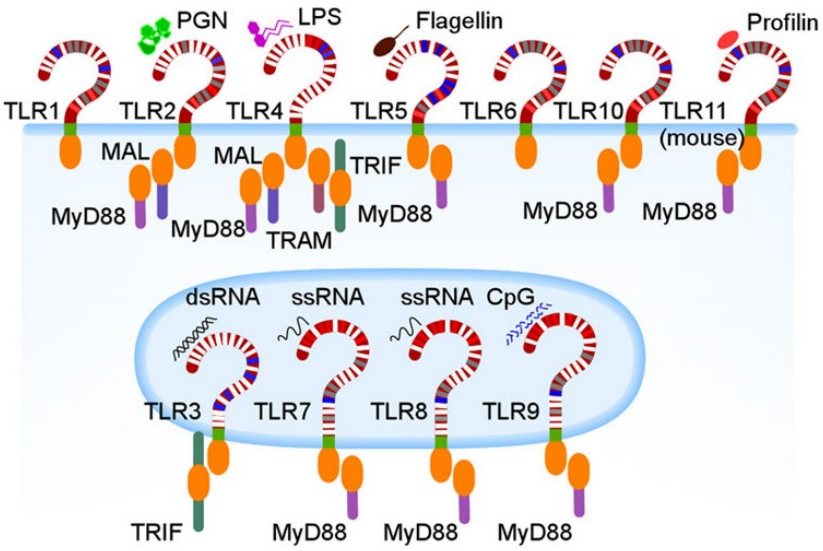


Figure 5 *- Mammalian TLRs, their ligands and adaptors.*
(http://labs.mmg.pitt.edu/sarkar/Signaling.htm).

TLR are specialized in pathogens recognition, and are expressed on both cell surface and endosomes. Different epithelial surfaces present different TLRs and this has a clear influence on the way the organism responds to different threats (e.g. bacterial, viral) in different areas (Hopkins *et al.*, 2005).

The distribution of TLRs in the intestinal epithelium is very sophisticated especially because there are different anatomical compartments in which there is a different pattern of expression of TLRs.

TLRs not only are specific according to the tissue in which they are located, but also their expression is not always static. In fact, the expression of TLRs mRNA is altered by several factors such as the age of the host and mostly the exposure of cells to environmental stress, bacteria and cytokines (Hopkins *et al.*, 2005).

Nucleotide oligomerization domain NOD-like receptors (NLRs) are a specialized group of intracellular proteins that are involved in the regulation of the host innate immune response, and the main function of these proteins is to recognize microbes in the cytosol (Kanneganti *et al.*, 2007).

Specific NLRs control the activation of inflammatory caspase-1 (evolutionarily conserved enzyme that proteolytically cleaves other proteins), which causes the maturation and secretion of IL-1β and IL-18. NLRs collect signals which induce platforms called inflammasomes, triggering NF-κB and mitogen-activated protein kinase signaling pathways (Kanneganti *et al.*, 2007; Franchi *et al.*, 2009).

Intestinal epithelial cells also express high levels of Interleukin-1 receptor 8 (IL1-R8, also known as SIGIRR or TIR8), a transmembrane and orphan receptor, that is a negative regulator of TLR and IL-1R signaling. This affects the communication among IECs, immune system and commensal bacteria (Garlanda *et al.*, 2009, Riva *et al.*, 2012).

In order to enable microbial colonization of the gut mucosa, the intestinal immune system needs to react to danger signals but also to recognize symbiotic microbiota, and it seems that, paradoxically, the recognition of both, pathogenic and commensal bacteria, is mediated by the same environmental sensors. So commensal bacteria inhibit NF-κB and produce metabolites that induce the expression of anti-inflammatory cytokines such as Interleukin-10 (Swiatczak *et al*., 2015).

Leukocytes trafficking to gastrointestinal tract

An important aspect of the mucosal immune system is the molecular mechanisms that regulate the trafficking of leukocytes in mucosal sites.

In adult animals, leukocytes are produced in the bone marrow, originating from precursor cells, there they mature and finally enter the bloodstream circulation. From the bloodstream leukocytes reach the secondary lymphoid organs or the tissues, such as the mucosal sites, following tightly controlled mechanisms.

The fine regulation of leukocyte trafficking in the gastrointestinal tract maintains intestinal immune homeostasis, mediates immune responses, and controls inflammation.

The recruitment of leukocytes to specific body compartment is mediated by a wide panel of receptors, chemoattractant and adhesion molecules expressed on leukocytes, epithelial cells, endothelial cells and stromal cells.

Different trafficking programs characterize the different leukocyte subpopulations and enable them to migrate into specific microenvironments.

While a great amount of data are available on the trafficking of leukocytes to the intestine, very few data are present in the literature about the trafficking of leukocytes in the upper tracts (esophagus, stomach and ruminant forestomachs). The following description of the trafficking of leukocytes will refer mainly to the small intestine and colon.

Monocytes and Neutrophils

Circulating monocytes and neutrophils represent the first line of defense against pathogens, but they are also involved in wound healing. Both monocytes and neutrophils do not express the α4β7 integrin nor the receptors for the mucosal constitutive expressed chemokines CCL25 and CCL28.

These myeloid cells are recruited in the gut exclusively under inflammatory conditions thanks to the activity of vascular selectins, L-selectin ligands, P-selectin glycoprotein ligand 1 and inflammatory chemokines, following the same mechanism of recruitment in all the other districts of the body (Fournier *et al*., 2012; Gerhardt *et al.*, 2015).

Dendritic cells

The dendritic cells (DCs) reside in the gastrointestinal tract and their task is to sample different antigens from diet, commensal and pathogenic bacteria. These cells are specialized antigen presenting cells (APCs) that transport and process the antigen from periphery to draining lymph nodes in order to present them to lymphocytes.

Human and murine dendritic cells of gastrointestinal tract present different specialized subsets. Each subset expresses characteristic trafficking and adhesion molecules and shows specific migratory properties (Cerovic *et al*., 2013).

Conventional DCs (cDCs) in the gut are characterized by the expression of the integrin CD103, that mediates the adhesion to E-cadherin (CDH1) of the epithelium with the help of β7 integrin, promoting the retention of DCs in the gut (Kilshaw, 1993). CD103 positive cells are divided into $CD11b^-$ (cDC1) and $CD11b^+$ (cDC2) cells (Watchmaker *et al*., 2014). cDC1 and cDC2 express different pattern of chemokine receptors suggesting a different micro environmental location in the gut. The two subsets of DCs express also different patterns of TLRs that enable them to respond to different microorganisms, but their activation leads to CCR7 upregulation and consequently to migrate to the draining lymph nodes (Watchmaker *et al*., 2014; Jang *et al*., 2006). cDC2 also express the lectin CD209 that is involved in the interaction with T cell ICAM3 and in the ICAM2-dependent rolling on the endothelium (Geijtenbeek *et al*., 2000).

Migrating DCs can process vitamin A to retinoic acid that is an important molecule during antigen presentation to the T cells specifically homing to the small intestine (Kim *et al.,* 2011).

Some DC subsets express CCR6 that induce their migration to the peyer's patches (PP) subepithelium expressing the chemokine CCL20. This DC subset localize under the PP dome where they can sense the antigens captured by M cells (Salazar-Gonzalez *et al*., 2006).

CD103 negative subset of DCs expresses CX3CR1 and is mainly resident cells that do not migrate to PP or lymph nodes (Varol *et al*., 2009).

The trafficking of plasmacytoid DCs is mainly regulated in mice by CCR9 and in humans by CMKLR1, but the fine mechanisms are still unknown (Wendland *et al.*, 2007; Zabel *et al.,* 2005).

Naïve and memory lymphocytes

Naïve and memory lymphocytes circulate in the bloodstream and reach the lymph nodes or the GALT through the high endothelial venules (HEVs). GALT HEVs express high levels of mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), a receptor for the α4β7 integrin present on lymphocyte surface (Streeter *et al*., 1998). MADCAM1 can also bind L-selectin (CD62L) and the B cells lectin Siglec2 (Lee *et al.,* 2014).

Lymphocytes activated by retinoic acid produced by DCs in the mesenteric lymph nodes up-regulate α4β7 integrin (Agace *et al.,* 2012).

HEVs in GALT, Peyer's Patches and mesenteric lymph nodes express other adhesion and chemotactic molecules such as CCL21, CXCL12, CXCL13, peripheral lymph node addressin, involved in the recruitment of naïve and central memory T and B cells (Okada *et al.*, 2002; Michie *et al.*, 1993).

The interaction/binding of these HEV molecules with α4β7 integrin and other adhesion molecules expressed by lymphocytes promotes their tethering and rolling. In particular the engagement of MADCAM1 with α4β7 integrin slows the rolling of lymphocytes allowing them to respond to the chemotactic signals and promoting the arrest mediated by ICAM1 and LFA-1 (Bargatze *et al.,* 1995).

Peyer's patches (PPs) are populated by a large number of B cells that are ready to sense intestinal antigens, differentiate to gut-homing plasmablast that produce secretory IgAs (Mora *et al.*, 2008). B cells show a high affinity to PP HEVs dependent on L-selectine and α4β7 integrin. The arrest of B cells into HEVs is mediated by chemokines and their receptors: CCL21/CCR7, CXCL12/CXCR4, CXCL13/CXCR5 (Okada *et al.,* 2002). CXCL13 in particular allows a selective adhesion of B cells in HEVs (not for T cells), representing a local micro environmental control of lymphocyte-endothelial interaction (Warnock *et al.*, 2000). A similar role is played by CD22 and its endothelial ligand predominantly expressed in PP HEVs (Kimura *et al*., 2007).

Naïve and central memory lymphocytes stay in lymphoid tissues searching for antigens. Once they encounter a specific antigen, migrate to the tissue where their action is needed.

The migration of T cells, including Treg, to intestinal epithelium is mediated by α4β7 integrin and CCR9 (Guy-Grand *et al*., 2013). Indeed intestinal epithelium expresses CCL25, the CCR9 ligand and the lamina propria venules express MADCAM1, the α4β7 integrin ligand (Kunkel *et al*., 2000). Some T cell subset direct to intestinal epithelium using only α4β7 (naïve CD4) or GPR18 (CD8-aa) (Wang *et al*., 2014). This mechanism is predominant in the proximal small intestine,

while in the distal small intestine and in the colon CCR9-indipendent mechanisms are involved (Kunkel *et al.*, 2000).

B cells activated in PPs exit within the lymph under the control of CXCR5, CXCR4 and G-protein–coupled receptor sphingosine-1 phosphate receptor 1 (Kunkel *et al*., 2003). CCR6 drives memory B cells to inflammatory sites, helped by α4β7 integrin and CCR9 (Mora *et al*., 2009).

A subset of bovine γδT cells, also positive for CD8, were recently demonstrated to express high levels of α4β7 integrin but low levels of L-selectin and E-selectin. These cells selectively migrate in vitro to CCR7 ligands CCL21 and CCL19. Moreover, CCL21 stimulation significantly increased the binding to MADCAM1. This data suggest the capability of bovine γδT $CD8^+$ cells to accumulate in mucosal sites also in absence of exogenous Ag stimulation (Wilson *et al.*, 2002).

Another study demonstrated that in tonsils, but overall in PPs, the homing of lymphocytes is regulated by the interaction of α4β7 integrin/MADCAM1 (Rebelatto *et al*., 2000).

Recent studies demonstrated that also in the upper regions of the gastrointestinal tract the trafficking of leukocytes is regulated by similar molecular mechanism.

In particular, preliminary studies at a gastric level shown that CCL25 (a cognate ligand for CCR9) is not expressed in gastric mucosa, and do not support the hypothesis that gastric DCs induce T-cell α4β7 expression, this means that at a gastric level the way DCs may induce T-cell homing is still unknown (Bimczok *et al*., 2015).

The rumen is one of the first organs that comes in direct contact with ingested feed particles, microorganisms and metabolites and can react to noxious components, acting as a first defense mechanism. Few studies about mucosal immune and inflammatory response in the rumen are available. One study from Reynolds *et al.*, 2017, shows different gene expression in the rumen of two chemokines (CCL11, CXCL5) and one receptor gene (IL10RA) among beef cattle with divergent average daily gain, showing a different predisposition of the inflammatory response in the rumen papillae. It was proved that CCL11 (that has chemotactic specificity for eosinophils) expression increases with the presence of RNA virus infections, and seems to be involved in episodes of asthma and allergies (Ponath *et al*., 1996). CXCL5 is expressed as a result of cell stimulation by cytokines, and in the rumen it is involved in neutrophil recruitment (Zhou *et al*. 2012).

Acute phase response

The immune response even at mucosal sites can lead to a systemic acute phase response.

The acute phase response (Fig. 6) is a complex of systemic innate defense mechanisms seen in response to trauma, infection, stress, neoplasia, and inflammation, that is characterized by changes in the production of hepatic-derived blood proteins (acute phase proteins (APPs)) (Armstrong *et al.*, 1999; Raida *et al*., 2009).

Inflammatory cytokines produced during innate immune responses (e.g. tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6)) are released at the site of injury and through the blood they reach the liver where hepatocytes synthesize and secrete APPs; these are circulating blood proteins, and depending on their increase or decrease can directly or indirectly affect the immune response (Moshage, 1997; Gabay *et al*., 1999).

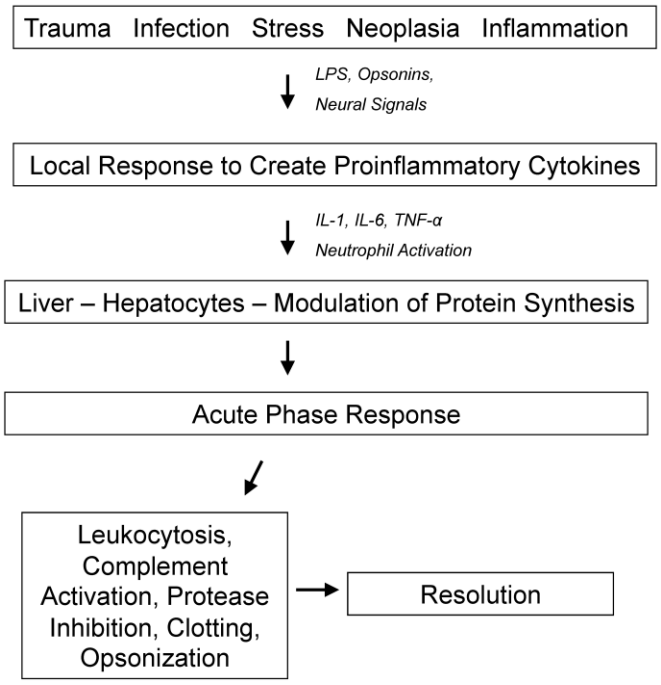


Figure 6 *- The acute phase response (Cray et al., 2009).*

APPs can be divided into positive and negative, that means their levels increase or decrease, respectively, in response to a stressor. Among APPs in cattle, we can distinguish some negative APPs (e.g. Albumin and transferrin), from those considered positive (e.g. haptoglobin (Hp), serum amyloid A (SAA), and fibrinogen) (Murata *et al*., 2004).

Albumin represents the major negative APP in almost all animal species. During the acute phase response, albumin concentration decreases in bloodstream and this may be either because of a selective loss of albumin due to renal or gastrointestinal changes or of a decrease in the hepatic

synthesis of the APP (Kaneko *et al.* 1997). This change does not seem directly connected to the acute phase response, but allows for the use of amino acids in the liver to produce positive APPs and other important mediators of inflammation (Paltrinieri, 2008).

Positive APPs increase during an acute phase response, and can be classified as major (they markedly increase in the first 48h after the triggering event and have a short half-life), moderate, or minor (normally associated with cases of chronic inflammation), depending on the magnitude of their increase (Petersen *et al*., 2004; Ceron *et al*., 2005).

Among the most studied acute phase proteins we can find:

- C-reactive protein (CRP) that can function as an opsonin by binding specific molecules on microorganisms, activating the classical complement pathway or phagocytosis. In addition, CRP can also upregulate or downregulate cytokine production and chemotaxis. However, CRP is not commonly used in cattle because its role as APP is dubious.

- Serum amyloid A (SAA) can act as stimulus for the chemotaxis of monocytes, polymorphonuclear cells, and T cells, and it can also downregulate the inflammatory process;

- Haptoglobin (Hp) binds hemoglobin and can reduce oxidative damage associated with hemolysis. It also has bacteriostatic and immunomodulatory functions. In cattle, Hp is usually considered a second phase APP, with peak levels at 7-10 days post inflammation;

- Mannose-binding lectin (MBL) binds to mannose-rich glycans on microbial cell walls, acting as an opsonin, promoting this way phagocytosis and activating the complement lectin pathway;

- The α1-acid glycoprotein binds LPS and it's able to inhibit its activity, and it can also downregulate neutrophils and complement activity;

- α2-macroglobulin has protease inhibitory activity and is important in removing enzymes released during injury;

- Ceruloplasmin scavenges free radicals;

- Fibrinogen provides a substrate for fibrin formation, improving tissue repair, and is used in cattle as an indicator of bacterial infection, inflammation, and surgical trauma. Similarly to Hp, also fibrinogen is considered a second phase APP in cattle (Morimatsu, *et al*., 1991; Mackiewicz, 1997; Hirvonen *et al*., 1998; Johnson *et al*., 1999; Suffredini *et al.*, 1999; Murata *et al*., 2004; Petersen *et al*., 2004; Ceron *et al.* 2005).

SAA and Hp have been identified as the major positive bovine APPs, which can increase several folds after tissue injury in cattle, but this happens in acute rather than chronic inflammatory conditions, while α1-acid glycoprotein levels are more likely to be elevated during a chronic inflammation situation. On the other hand, Hp has been recognized as a valuable marker of disease

in ruminants, providing additional information to the traditional hematological studies (Skinner *et al*., 1991; Murata *et al*., 2004).

APPs had also been described as potential biomarkers of stress (Murata *et al*., 2004; Schrödl *et al*., 2016). Although the mechanism of APP induction in response to stress is yet to be understood.

Stress and immune system

Stress occurs when the organism identifies a threat of disturbance of its own homeostasis (NRC, 2008).

There are different kinds of stressors, that can activate different response mechanisms in the organism, including behavioral reactions (behavioral response), activation of the sympathetic branch of the autonomic nervous system (ANS) (nervous response), activation of the hypothalamic-pituitary-adrenal (HPA) axis and adrenal gland, and secretion of stress hormones (neuroendocrinal response), and activation of the immune system (immune response) (Minton, 1994). Those mechanisms can act alone or together, but none of them is exclusive to identify a stressful state (Moberg, 2000; Schneiderman *et al.*, 2005).

In a response to stress, and in order to return to its homeostatic state, the organism first activates behavioral mechanisms, specific for the organism of different species or even of the same specie but with different genetic backgrounds (Hall *et al.*, 1998). A really interesting feature about it is that the organism may not always manifest stress with recognizable evident behaviors traces but, instead, sometimes with subclinical pathological changes (Ghasemian Karyak *et al.*, 2011).

When looking into the endocrine response to stress, the HPA axis is one of the primary systems that reacts (Chrousos, 2000; Moberg, 2000). In response to a stressful situation, the hypothalamus produces corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). Those hormones travel to the anterior pituitary and stimulate the release of adrenocorticotropic hormone (ACTH) into the circulatory system (Antoni, 1993). In its turn, ACTH acts on specific receptors in the adrenal cortex, stimulating the release of steroids that promote the uptake of cholesterol and its enzymatic conversion to glucocorticoids (cortisol and corticosterone) (Fig. 7). Glucocorticoids have a key role in gluconeogenesis since they are involved in the conversion of glycogen into glucose, and promote lipolysis making fat useful as energy source (Brindley *et al.*, 1989). And finally, when the stressor is removed, glucocorticoids bound to receptors in the hypothalamus and anterior pituitary initiating a negative feedback that causes a decreasing in the production and release of CRH and ACTH, stopping this way the hormonal response (Keller-Wood, 2015). Unfortunately, the fact that glucocorticoids mobilize energy in response to a stress situation can have negative

consequences in the cardiovascular system (e.g. hypertension, hyperglycemia, and obesity), gastrointestinal system (e.g. increase risk of gastritis, peptic ulceration, gastrointestinal hemorrhage), central nervous system (CNS) (e.g. alterations of mood, memory deficit, psychosis), musculoskeletal (e.g. osteoporosis) and endocrine glands (e.g. hyperlipidemia and hyperglycemia) (Moghadam-Kia *et al.*, 2010).

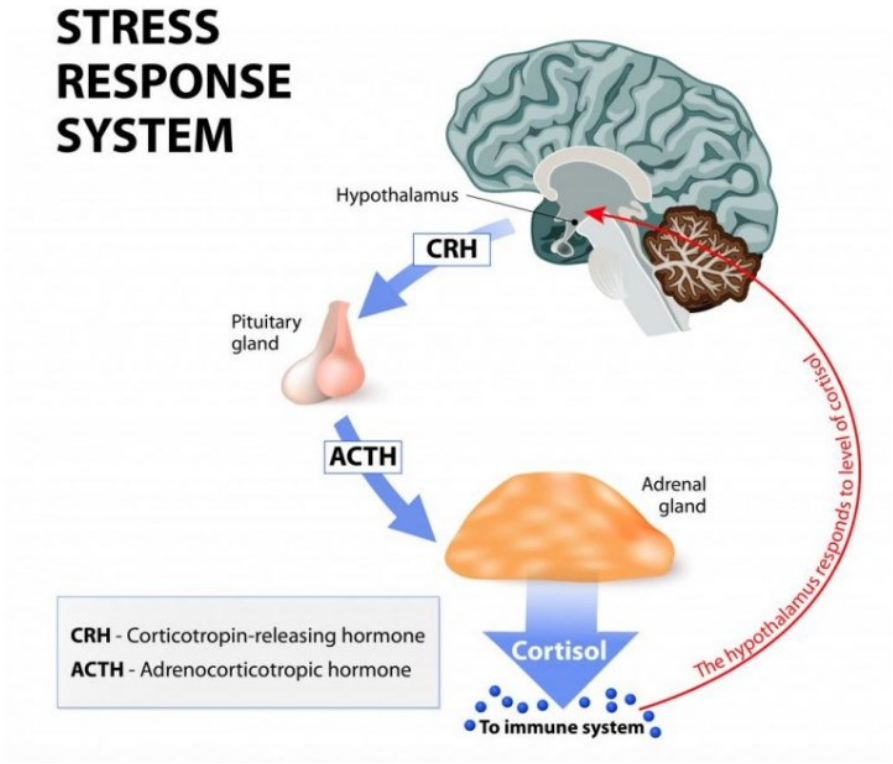


Figure 7 *- Endocrine response to stress.*
(http://barefoothealth.me/do-you-have-adrenal-fatigue/hpa-axis-1024x962/)

An important part of the sympathetic harm of the ANS, the adrenal medulla, works as an endocrine organ and plays a pivotal role in the overall regulation of the HPA axis. The activation of the sympathetic pathway initiates with the release of an hormonal cascade from the adrenal medulla that results in the secretion of catecholamines (e.g. adrenaline, that is the main hormone secreted by the adrenal medulla, and noradrenaline), which can act on various target organs and tissues, and stimulate CRH secretion, preparing the animal to react to the stressor. While both adrenaline and noradrenaline increase alertness and awareness, serotonin seems to be associated with anxiety and fear (Fell *et al.*, 1985; Abelson *et al.*, 1996; Arroyo *et al.*, 2016). This activation leads to increased heart rate, blood pressure, breathing, and increased mobilization of nutrients, and redistribution of bloodstream (Blanc *et al.*, 1991). Some stressors may also increase the activity of the parasympathetic partition, affecting body temperature and the gastrointestinal system (Goldstein, 2013).

It is important to recognize that there is a bidirectional communication between the immune and neuroendocrine systems, with the two systems sharing a common set of hormones and receptors (Taub, 2008). This means that the immune system is under the control of hormones, which can play an important role as modulators of the immune system (Cutolo *et al.*, 2004). Examples of that are metabolic hormones (e.g. growth hormone, thyroid stimulating hormone, insulin), which are essential for the development of the immune system and its functions, and the hypothalamic-pituitary-adrenal axis, which has a suppressive role in the presence of long term or chronic stressors (Sapolsky *et al.*, 2000; Taub, 2008).

Is now known that IL-6 synthesis in macrophages is controlled by the CRH-DHEA (Corticotropin-releasing hormone – Dehydroepiandrosterone) circuit (Squire *et al.,* 2009), and that IL-1 is capable of inducing the activation of the hypothalamopituitary-adrenocortical axis as well as stimulate the expression of cerebral noradrenaline (Dunn *et al*., 1999). Also, the brain can produce IFN-α in response to stress; and this cytokine may change the brain activity in order to have a feedback effect on the immune system (Hori *et al*., 1998).

Different signaling pathways link the brain to the immune system, combining neuroendocrine and immune system activation (Dhabhar, 2009; Nance *et al.,* 2007). During a stressful situation, lymphocytes and macrophages are activated (e.g. by IFNγ produced by NK cells) and are able to express receptors for different hormones and neurotransmitters (e.g. adrenoceptors, which translate neuronal signals into immune cell signals) (Carr *et al.,* 1991; O'Connor *et al.*, 2003; ThyagaRajan *et al.*, 2012). And it has also been demonstrated that cytokines and other humoral mediators of inflammation act as important activators of the central stress response. Three main inflammatory cytokines – TNF-α, IL-1, and IL-6 – are able to increase the synthesis and secretion of CRH and AVP at the hypothalamus (Naitoh *et al.*, 1988; Bernardini *et al.*, 1990).

In mice it has been demonstrated that the control of mental stress is dependent on peripheral immunity. A short exposure to a mental stressor can increase T-cell infiltration to the brain, that is associated with an increased ICAM-1 expression by choroid plexus cells. The mental stress response can be reduced by immunization with a CNS-related myelin peptide (Lewitus *et al.*, 2008). This seems to mean that a stress response activates a protective adaptive immune response to self-tissue antigens, and this activity is regulated by $CD4^+$ $CD25^+$ T cells (Cohen *et al.,* 2006).

Metabolic stress is a peculiar type of stress that induces instability in the homeostasis of the organism. In this situation the body does not use nutrients properly, and the demand for calories and

proteins increases. The main cause of metabolic stress is the deficiency of nutrients in the environment (Dancsó *et al.*, 2010). This condition may be highly dependent on seasonal variations, but in domesticated animals, like dairy cows, metabolic stress is mainly due to either deficiency or excess of nutrients, and frequently it is related on pathological or physiological conditions, which may alter nutrient intake, assimilation and/or incorrect feed utilization (Wellen *et al.*, 2010; Carlton *et al.*, 2014; Sordillo *et al.*, 2014).

It is now clear that metabolic stress provokes also a stress response, which interferes with the neuroendocrine response causing an increase of the glucocorticoid levels in the body. Metabolic stress can modulate both innate and acquired immune responses (Hotamisligil *et al.*, 2006). The best example is represented by periparturient dairy cows (Lacetera *et al.*, 2010).

For livestock, and especially for high-yielding dairy cows, the periparturient period (also called transition period, that was defined by Grummer, 1995 and Drackley, 1999 as the interval from 3 weeks before to 3 weeks after calving) represents a critical phase of metabolic stress risk. Indeed, in this period cows are submitted to many challenges that require rapid and efficient adaptive responses. These challenges can be biotic (e.g. bacteria, virus, parasites) and abiotic (Amadori, 2016). The abiotic causes, that can also be defined as non-infectious, are actually quite frequent and are particularly relevant in this period. These include psychological stresses such as isolation or overcrowding (Loor *et al.,* 2013), lactation onset and related oxidative stress (Celi, 2011; Sordillo *et al.,* 2014), nutritional features (Loor *et al.*, 2013; Bertoni *et al.*, 2016), and unfavorable environmental conditions.

Different studies identified five critical aspects of the periparturient period in dairy cows:

- reduction of immune competence (Goff *et al.*, 1997; Mallard *et al.*, 1998; Lacetera *et al.*, 2005);
- oxidative stress (Bionaz *et al.*, 2007; Sordillo *et al.*, 2009a; Celi, 2011);
- hypocalcemia (De Garis *et al.*, 2009; Martinez *et al.*, 2012);
- negative energy balance (Grummer, 1995; Drackley, 1999);
- an overt systemic inflammatory response around the time of calving (Bionaz *et al.*, 2007; Sordillo *et al.*, 2009a; Trevisi *et al.*, 2012; Bertoni *et al.*, 2013).

In the scientific literature, we can find evidence of a correlation between metabolic stress and a systemic inflammatory response that plays a significant role in early lactation, affecting many liver functions and often reducing animal performance (e.g. reduced feed intake, reduced milk production, reduced fertility, reduced welfare, etc.) (Sordillo *et al.*, 2014). These conditions are

evident in high-yielding dairy cows, like Holstein-Friesian around calving.

The immune system is clearly involved in this process, and many changes of the cow's immune system parameters have been observed around calving; for example, different studies suggest that the immune system is clearly dysregulated around parturition. Till now, major consideration has been focused on cellular functions (e.g. the reduction of phagocytosis and leukocyte extravasation), but new studies start to take into account susceptibility to inflammatory events and the related acute phase response (Amadori, 2016).

Some studies also demonstrated that in dairy cows, overconditioned animals before parturition have more metabolic disturbances and this is one of the most important risk factors for these animals to develop health problems during lactation or to experience production losses, immunosuppression, and decreased fertility (Torres-Rovira *et al.*, 2011; Carlton *et al.,* 2014). On the whole, it appears that in transition dairy cows, obesity simply aggravates a physiological predisposition of all high-yielding subjects to mobilize fat from the adipose tissue during early lactation, since lipid mobilization is necessary to respond to the high energy demand for milk production (Lacetera *et al.*, 2010). Thus, weight loss and increase of plasma nonesterified fatty acids (NEFA) due to lipomobilization represent common findings in high yielding, early lactating dairy cows (Lacetera *et al.*, 2005; Leroy *et al.*, 2005). However, human and rodent studies demonstrated that lipomobilization can affect directly and indirectly leukocyte and endothelial inflammatory response (Zhang *et al*., 2006; Contreras *et al.*, 2011). Studies carried out in sheep and cows demonstrated that NEFA may also impair both mononuclear and PMN leukocytes functions. For example, an increase of NEFA increases monocyte adhesion to endothelial cells, this adhesion requires interaction of integrins such as the β2 integrin Mac-1 (CD11/CD18) with ICAM-1 on the surface of endothelial cells, and NEFA is capable of stimulating the expression of both message and protein for CD11b (Albelda *et al*., 1994; Lacetera *et al.*, 2001; Lacetera *et al.*, 2002; Lacetera *et al.*, 2004; Scalia *et al.*, 2006; Zhang *et al.,* 2006; Contreras *et al.*, 2011).

Different studies documented that periparturient dairy cows, and in particular animals with higher body conditions score (BCS), also experience a variable degree of insulin resistance in peripheral tissues, hepatic lipidosis, are more prone to oxidative stress, and have higher plasma reactive oxygen metabolites (ROM) (high levels of ROM cause significant damage to cell structures, with DNA and RNA damage, lipid peroxidation, etc.), thiobarbituric acid-reactive substances (TBARS) (that are a result of oxidative degradation of lipids) and thiol groups (SH) (Bernabucci *et al.*, 2005; Bernabucci *et al.*, 2009; Leiva *et al.*, 2014).

Oxidative stress is essentially an imbalance between the production of free radicals and

antioxidants. Is well knows that all organisms maintain a reducing environment within their cells; this environment is preserved by enzymes that are able to do it through a constant input of metabolic energy. Any disturbances in this state may have toxic effects for the production of peroxides and free radicals that can damage the cell (including proteins, lipids and DNA) (Kala *et al*., 2015).

Nevertheless, there are several other factors, which may increase prooxidants, decrease antioxidants, or do both, and that may contribute to oxidative stress. Among these, a number of environmental pollutants (e.g., heavy metals), heat stress, exposure to mycotoxins (secondary metabolites produced by molds), and deficiency of specific antioxidant nutrients (Bernabucci *et al.*, 2011; Fittipaldi *et al.*, 2014; Liu *et al.*, 2015) contribute to worsen the oxidative stress.

It is now clear that an extreme or persistent immune response may also alter the metabolic status and nutrient requirements (Husband *et al.*, 1996). This may lead to important consequences like fever, lack of appetite, amino acid resorption from muscle, and redirection of nutrients toward liver anabolism of acute phase proteins (Colditz, 2002). Consequently, under chronic activation of the immune system a reduction of productive and reproductive performances may take place in dairy cows (Amadori, 2016). However, the fine mechanisms underlying physiological (immune and metabolic) variations in the transition period have not yet been identified.

The innate immune system provides the first line of host defense against bacterial or viral infections. Recent evidences indicate that the innate component of the immune system, similarly to the adaptive immune system, can develop a memory-like behavior, and this evidence completely changes the way we look at its functions (Bordon, 2014). Innate immunity provides immediate non-specific defense to the host against both infectious and non-infectious stressors recognizing specific molecules (e.g. PAMPs and DAMPs) through PRRs. PRRs activation is essential to trigger an immune response, and it can either lead to phagocytosis and direct elimination of the pathogen or promote the secretion of soluble mediators such as proinflammatory cytokines, that will lead to the recruitment of leukocytes to the infection site (Cheng *et al.*, 2014).

DAMPs (Damage-associated molecular patterns) are self-molecules, that can initiate a “sterile inflammatory response”. The fact that PRRs are capable to identify DAMPs provides an explanation for how tissue injuries could result in a “sterile inflammation”, which has a lot of similarities with the inflammation process during a bacterial infection (Amadori, 2016).

Examples of well-known DAMPs that can activate the inflammatory cascade are S100 protein,

uric acid, high mobility group box 1 (HMGB1) protein or amphoterin, and heat shock proteins (HSPs) (Basu *et al.*, 2000; Rosin *et al.*, 2011).

A study from Aneja *et al.*, 2006 demonstrated that HSP70 is released into the extracellular space after cellular stress, and appears to act as a danger signal to the innate immune system, activating it by a CD14-dependent mechanism, and with the participation of TLR 4.

Other studies suggest a variety of other signals that may "wake up" the innate immunity system after exposure to non-infectious stressors, and those can include catecholamines, glucocorticoids, and even metabolites of commensal gut microbiota (Mazzeo *et al*., 2001; Johnson *et al.*, 2005; Frank *et al.*, 2012; Maslanik *et al.*, 2012). However, their role under conditions of metabolic stress has not yet been studied.

Studies on mice and humans have demonstrated the existence of two main PRRs signaling pathways (mTOR- (mechanistic target of rapamycin) and eIF2a-dependent (eukaryotic initiation factor 2) signal transduction cascades) that relate nutrient availability, metabolic stress and innate immunity (Wek *et al*., 2006; Laplante *et al*., 2012). The signaling pathway mediated by mTOR, that is an atypical serine/threonine kinase regulated by IFNs, is essential in the regulation of cellular metabolism (Liu *et al*., 2017b). mTOR interacts with quite a few proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is the better characterized of the two complexes and integrates inputs from different intracellular and extracellular signals, including stress, and it is involved in many processes, including protein and lipid synthesis and autophagy. Some proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), can activate mTORC1 through a mechanism conceptually similar to growth factors (Liu *et al*., 2017b).

In response to environmental stress eIF2 becomes phosphorylate by protein kinases, and this phosphorylation of eIF2 reduces global translation, allowing cells to conserve resources and to initiate a reconfiguration of gene expression to effectively manage stress conditions. eIF2 phosphorylation is also responsible for the translation of specific mRNAs (e.g. bZIP (basic leucine zipper), ATF4 (activating transcription factor 4), etc.), and some of these are important in the regulation of genes involved in metabolism, in the redox status of the cells and in apoptosis. Reduced translation by eIF2 phosphorylation can also lead to activation of stress-related transcription factors, such as NF-κB (nuclear factor κB) (Wek *et al.*, 2006).

But also TLR4, a main receptor for LPS, has been linked to the development of inflammation connected with a state of hyperlipidemia (e.g. NEFA may contribute directly to greater inflammation by binding TLR4 and initiating an inflammatory cascade through TNF-α), and other

molecules just like HSPs, when released into the extracellular environment, can act as alarm signals to the immune system (Song *et al.*, 2006; Hotamisligil *et al*., 2008; Fleshner *et al.*, 2013). Specific fatty acids, particularly saturated fatty acids, appear to trigger TLR, which provides an additional source of inflammation (Ingvartsen *et al.*, 2013). NEFA activate TLR4, which activates in turn NF-κB and leads to secretion of TNF-α, IL-1beta and IL-8. TNF-α and IL-1 act on intracellular messengers to up-regulate inflammation and increase insulin resistance (Osborn *et al*., 2012).

Finally, it deserves to be mentioned that NOD-like receptor proteins also trigger the inflammasome reaction in response to the energy stress caused by metabolic inhibitors (e.g. 2-Deoxy-D-glucose) or nutrient deficiency. The inflammasome assembly results in caspase-1 activation and cleavage of pro-IL-1β and pro-IL18 into their biologically active forms (Fleshner *et al.*, 2013; Liao *et al.,* 2013).

Owing to the above, metabolic stress conditions are directly perceived by the immune system. The molecular mechanisms behind the activation of the innate immune response in animals suffering from metabolic stress are diverse, and the physiological significance of such activation has not yet been fully understood. The other way round, immune activation has several metabolic implications and it is now clear that excessive/prolonged inflammatory conditions may also alter metabolic status and nutrients requirement, which may be responsible for the reduction of productive and reproductive performances, metabolic diseases, immune suppression, and outbreaks of infections in dairy cows (Amadori, 2016).

The evaluation of immune-metabolic indices may offer opportunities to predict the onset of the health troubles and perhaps anticipate proper therapies needed to guarantee health and welfare. In practice, some components of the innate immune system could be meant as risk signals when critical thresholds are exceeded, not only during infection, but also during exposure to environmental, non-infectious stressors (Trevisi *et al.,* 2017).

Forestomachs pathological conditions and their immune response

As already described previously, the immune response in the mucosal districts is orchestrated by MALT, that is actually called GALT in the gut. The main activity of GALT relies on the capability of mucosal epithelial cells to discriminate between pathogenic and commensal bacteria. Such recognition is essential for the development and function of the immune system in the mucosal and peripheral districts (Macpherson *et al.*, 2004). Signals provided by commensal bacteria

determine a basal level of activation that promotes gene expression patterns required for normal development and function of immune cells (Abreu *et al.*, 2001; Rakoff-Nahoum *et al.*, 2004; Uehara *et al.*, 2007).

Unfortunately, till now scanty data are available about the organization of the mucosal immune system in bovine forestomachs (Dommett *et al.*, 2005), but since these functions are crucial for the global homeostatic balance in cattle, it is reasoned that epithelial cells in forestomachs could display mechanisms similar to those of the intestinal tract, in order to discriminate between commensal and pathogenic bacteria, as well as to recognize dangers associated to abnormal fermentations of ruminal bacteria (Trevisi *et al.,* 2009). This implies that epithelial cells of forestomachs can react to disturbances of the fermentation processes by releasing inflammatory and chemotactic molecules, intended for signaling at both local and systemic levels. Interestingly, it has been shown that inflammatory responses in forestomachs can be sustained by infiltrating leukocytes secreting cytokines in the rumen liquor; these can dramatically affect inflammatory and metabolic parameters in dairy cattle (Trevisi *et al.,* 2009).

Accordingly, production diseases can be accounted for by a dynamic relationship between metabolic disorders and effector activities of the innate immune system. In particular, production diseases of dairy cows include several pathologies, such as hypocalcemia, hypomagnesemia, ketosis, acidosis and Sub-Acute Ruminal Acidosis (SARA), abomasal displacement, metritis, retained placenta and laminitis, which mainly occur in the pregnancy to lactation transition period. These pathological conditions are considered man-made problems, mostly reported in the period of negative energy balance (NEB), when high-yielding dairy cows are unable to achieve a feed energy intake matching their high production requirements (Drackley *et al.*, 1999; Gozho *et al.*, 2007; Mulligan *et al.*, 2008). This is a common problem for Holstein Friesian dairy cows that have been genetically selected and managed for high production levels, and are highly susceptible to infectious and non-infectious stressors, among which the environmental ones undoubtedly play a relevant negative role, conducive to culling (Trevisi *et al.*, 2011; Van Knegsel *et al.*, 2014).

In recent years, many efforts have been devoted to a timely detection of Sub-Acute Ruminal Acidosis (SARA), also known as chronic or sub-clinical acidosis, a dangerous pathological condition and an increasing health problem in most dairy herds (Gozho *et al*., 2007). Dairy herds experiencing SARA have a decreased milk production efficiency, impaired health conditions and high rates of culling, which causes elevated economic losses (Abdela, 2016).

Results from different field studies indicate a high prevalence of SARA in high-yielding dairy herds, since farmers respond to the demands for increased milk production with higher grain and

lower fiber diets in order to maximize energy intake during early lactation (Kleen *et al.*, 2003; Abdela, 2016).

The biggest challenge nowadays is to implement feeding management and husbandry practices that prevent or reduce the incidence of SARA, even in high-producing dairy herds, where higher levels of concentrate are fed to maximize energy intake (Kleen *et al.*, 2004).

As for the diagnostic implications, SARA is a disorder of ruminal fermentation, characterized by ruminal pH below 5.5-5.8 for extended periods (Garrett, 1996; Oetzel *et al.*, 2000). For optimal ruminal fermentation and fiber digestion, ruminal pH should lie between 6.0 and 6.4, although, even in healthy cows, ruminal pH is likely to fluctuate below this level for short periods during the day. This drop in ruminal pH is a result of the breakdown of dietary carbohydrates, particularly from cereal grains. Grains have high contents of readily fermentable carbohydrates that are rapidly broken down by ruminal bacteria, leading to the production of volatile fatty acids (VFA) and lactic acid. Under normal feeding conditions, VFA are readily absorbed by the papillae on the rumen wall. Once absorbed, VFA enter the cow's bloodstream and can be used for milk production (Nordlund *et al*., 1995; Kleen *et al.*, 2003; Chilliard *et al.*, 2009).

SARA results from excessive VFA production that exceeds the ability of the ruminal papillae to absorb them. VFA accumulate in the rumen and, as a result, reduce ruminal pH. SARA typically occurs at calving when cows are abruptly switched from high fiber diets to higher concentrate, milking cow diets. An abrupt dietary change does not provide ruminal bacteria and ruminal papillae with adequate time to adjust, thereby leading to a rapid production and accumulation of VFA. Another common cause of SARA are improperly balanced or mixed rations, in which effective fiber content falls below recommended levels or fiber particle size is too small. This inhibits rumination and the production of saliva, which can buffer changes in ruminal pH (Nordlund *et al.*, 1995; Kleen *et al.*, 2003; Chilliard *et al.*, 2009).

Although cows experiencing SARA do not often exhibit clinical symptoms, common signs include reduced/erratic feed intake, reduced rumination, mild diarrhea, foamy feces containing gas bubbles, and undigested grain in feces. These signs however often go unnoticed in individual cows experiencing SARA, particularly in large dairy herds where cows are housed and fed in groups (Plaizier *et al.*, 2008).

In the long term, dairy herds experiencing SARA usually exhibit secondary signs of the disease including laminitis (Enemark *et al.*, 2002), low body condition (Oetzel *et al.*, 2000), ruminitis (Enemark, 2008), low milk fat syndrome and abomasal displacement (Olson, 1991). For this reason SARA should always be investigated as a cause, if the secondary signs are occurring for no

apparent reason. However, diagnosis in the field is complicated; indeed, the clinical signs are neither evident (subclinical cases), nor pathognomonic (Kleen *et al*. 2003; Abdela 2016).

Nowadays, depressed milk-fat content is still used as a diagnostic tool for SARA. The basis for this is that low pH inhibits fiber digestion in the rumen and the end products of fiber digestion are necessary for milk fat synthesis. The usual milk fat content in Holstein dairy cows is around 3.4-4%, so milk fat contents < 3% can indicate SARA. However, bulk tank testing of milk fat is inappropriate to diagnose SARA at herd level. Individual cows with SARA may have low milk fat values, but pooling their milk with that of the rest of the herd will mask the detection of SARA (Van Beukelen *et al.*, 1986; Garret*,* 1996; Oetzel, 2012).

Due to the above reasons, the most reliable diagnostic test for SARA is measuring ruminal fluid pH. While esophageal probes have been used on-farm to collect ruminal fluid samples for pH measurement, the results are often inaccurate due to contamination with saliva, which causes a non-specific increase of pH values. A practical method to obtain ruminal fluid samples under field conditions is a technique called rumenocentesis, that refers to percutaneous needle aspiration, and involves inserting a needle (16-gauge) into the ventral rumen sac, and taking a sample of ruminal fluid using a syringe. Because of its invasive nature, it is recommended that only a qualified veterinarian practitioner perform this procedure (Kleen *et al.* 2004).

But the reliability of ruminal pH measures remains controversial, and some of the signs attributed to SARA are also common dysfunctions related to other metabolic disorders (Penner *et al*., 2010; Plaizier *et al*., 2008; Trevisi *et al*., 2014b).

Ruminal disorders, ruminal acidosis and SARA are also known to be associated to an inflammatory response. Histamine LPS are present in increased concentrations in rumen of grain-fed dairy cattle (Gozho *et al*., 2007), and are suggested to trigger acidosis (Nagaraja *et al.*, 2007), possibly linked to laminitis (Nocek, 1997). LPS absorbed from rumen fluids could induce a modification of local blood circulation and coagulation, causing micro-circulation abnormalities and laminitis (Nocek, 1997). However, the latter hypothesis can be dismissed on the basis of the minimal plasma concentrations of LPS found during SARA, which confirm the efficient removal of LPS through the portal circuit even under acidosis conditions (Gozho *et al.*, 2007; Khafipour *et al.*, 2009). Finally, although an excess of rumen degradable protein causes an increase of ruminal histamine, the latter is physiologically non-relevant in terms of rumen pH and acidosis (associated to laminitis) (Pilachai *et al*., 2011).

In recent years, it has been proved that a clear correlation exists between metabolic status, inflammatory response, innate and adaptive immunity and resistance to microbial infections. In

dairy cows it is reported that around calving there is an overt systemic inflammatory response that can aggravate metabolic stress during the early lactation period (Sordillo *et al.,* 2009b; Amadori, 2016). Important consequences of immune stimulation include production of proinflammatory cytokines, activation of the acute phase response, fever, amino acid resorption from muscles, etc. Therefore, under chronic activation conditions of the immune system it makes sense that productive and reproductive performance be jeopardized in dairy cows (Amadori, 2016).

Owing to the above, the connections between diet disorders in cattle and occurrence of diverse disease conditions should be set into an alternative conceptual framework. This can be related to specific effector and regulatory roles of forestomach epithelial cells in the innate immune response (Dommett *et al*., 2005). Accordingly, previous studies indicated an active role of bovine forestomachs in the response to alimentary disorders as well as to inflammatory and infectious processes in both the gastro-intestinal tract and elsewhere (Trevisi *et al.,* 2009; Trevisi *et al.*, 2014a).

Indeed, the results presented by Trevisi *et al.*, 2014a revealed a modulation of the presence of IFN-γ and infiltrating leukocytes (at low concentrations) in rumen fluids of heifers submitted to different feeding regimes. In order to investigate how diet changes modify innate immune responses in rumen liquor, three rumen-fistulated heifers were submitted during 3 alternate experimental cycles to different feeding regimes: A) control (alfalfa hay + vitamin supplement); B) soy based feed (control + soy flour); C) maize based feed (control + corn flakes). The animals under study showed the same patterns of leukocyte infiltration during the control and soy-based diets, whereas a clear inhibition of the response was observed in all the animals that were fed the maize-supplemented diet, probably due to the lower pH levels caused by this diet, likely to be incompatible with leukocyte accumulation (Trevisi *et al.* 2014a).

In the same study, it was demonstrated that forestomachs walls are able to express both stimulatory and inhibitory receptors of the innate immune system, available for a cross-talk with infiltrating leukocytes. Indeed, it was detected the expression of IL-1R8 (TIR8/SIGIRR), Toll-like receptor (TLR) 4, IL-1β, IL-10 and Caspase-1 in the forestomach walls of healthy cows. Also, the presence of interferon (IFN)-γ, T and B lymphocytes, and myeloid lineage cells were detected in the ruminal content. This means that innate immune responses can be initiated, sustained and expanded in bovine forestomachs by an integrated system including receptors, signaling molecules, cytokines and infiltrating leukocytes (Trevisi *et al.* 2014a). In particular, these results indicate that bovine forestomachs can receive and elaborate signals for the immune cells infiltrating the rumen content, and participate in a cross-talk with the lymphoid tissues in the oral cavity, thus promoting

regulatory actions at both regional and systemic levels. This complex network is probably instrumental to the control of feed and diet quality, since uncontrolled inflammatory responses generated in the forestomachs can pose serious threats to animal health and welfare.

One way to reduce the prevalence of clinical cases and to increase animal welfare is to identify early diagnostic or prognostic biomarkers (Loor *et al.*, 2013). In this respect, a dysregulated activity of the immune system often precedes the typical negative energy balance around calving (Drackley, 1999) and the related reduction of plasma nutrients (i.e. amino acids, glucose and calcium) (Bionaz *et al.*, 2007; Zhou *et al.*, 2016), and increased concentrations of non-esterified fatty acids (NEFA) and ketone bodies (Bionaz *et al.*, 2007; Drackley, 1999).

Despite the significant improvement of both understanding and diagnosis of production diseases, their prevalence still remains high and causes severe economic losses to farmers (Trevisi *et al.*, 2014c). Among factors that trigger metabolic diseases, the changes of feed quality and feed intake during the transition period are very important (Sundrum, 2015).

Indeed, it is already known that diet can affect saliva production, rumen functionality, extent and profile of rumen fermentation, as well as the feed digestion and feed passage rates (Steele *et al*., 2009; Trevisi *et al.,* 2014a).

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 (Tajima *et al.*, 2000; Fernando *et al.*, 2010) caused by innate immune responses to metabolic stress (Amadori, 2016).

In this scenario, there is undoubtedly a need for new, early diagnostic markers of production disease risk in dairy cows.

Aims

In my PhD project, I postulated that the analysis of rumen fluids could be conducive to the identification of herds at risk for production diseases, in addition to the traditional analyses on blood and fecal samples.

The working hypothesis implied that ruminal fluids could be an important source of diagnostic information related to abnormal changes of ruminal contents and metabolites during metabolic stress and pathologic conditions, in agreement with the findings of a previous study (Trevisi *et al*., 2014a). In this rationale, it was my understanding to verify a possible role of the innate immune system in the metabolic alterations deriving from abnormal ruminal fermentations.

To this purpose, we first identified and characterized the populations of infiltrating leukocytes in the ruminal liquor, by both Flow Cytometry analysis and qPCR, and we also made a preliminary research about the origin of ruminal leukocytes.

The preliminary results provided the basis to make a field study. This field study was carried out in farms with animals without any clinical signs, but with rumen pH values that indicated a predisposition for SARA. From each farm, samples from 10 animals were taken, and with those samples we could verify a possible correlation between immune system parameters, production levels, and changes in the rumen liquor; we also investigated the differences among leukocyte populations of the rumen liquor from different herds. Later on, with the goal of developing a diagnostic kit that could highlight these new data, a large field survey of ruminal, fecal and blood parameters was performed in 12 different dairy farms of similar genetic merit.

Finally, we investigated the effects of a deliberate alteration of rumen fermentations with the use of a well-known antibiotic (Monensin), in order to verify if and to what extent the innate immune system reacts to a detectable change in ruminal metabolism.

Material and Methods

Saliva sampling and cell isolation

Saliva samples (5 mL/animal) were collected from 7 multiparous cows (4.0±1.7 calvings): 4 lactating cows (324±78 days in milk) and 3 dry cows (about 30 days before expected calving), hosted 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 minutes 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 (without Ca and Mg) 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.

Rumen, blood, and feces sampling

While the first and oldest technique used for studying and collecting ruminal fluid was the fistulation of the abdominal wall (Colin *et al*., 1854), nowadays, in Italy this technique is not allowed for diagnostic or zootechnical studies, being used only for scientific purposes. So this leaves us with two main techniques to collect rumen liquor for diagnostic purposes: esophageal probe and rumenocentesis.

In cattle the esophageal probe is a flexible plastic tube about 3.5 m long, to which a suction pump is attached. The probe reaches the rumen through the esophagus, (Nocek, 1997). This technique presents some disadvantages, indeed the pH value detected in this manner is not accurate, and depending on the location of the probe that is not always the same (reticulum, different areas of the rumen). Moreover the action of the pump in the rumen creates a turmoil that can alter the CO_2 content and saliva can contaminate the samples (saliva of cattle has a pH around 8, and even a small percentage of saliva can significantly alter the pH of the sample) consequently altering the pH of rumen fluid (Enemark, 2008).

Rumenocentesis, by the other hand, had been first described by Nordlund *et al*., 1994, and was subsequently modified by Gianesella *et al*., 2010; nowadays it is the most widely supported technique for rumen fluid sampling, because it provides the most accurate results (Garrett *et al*., 1999; Duffield *et al*., 2004; Morgante *et al*., 2007); for this reason it is considered the "gold standard" technique.

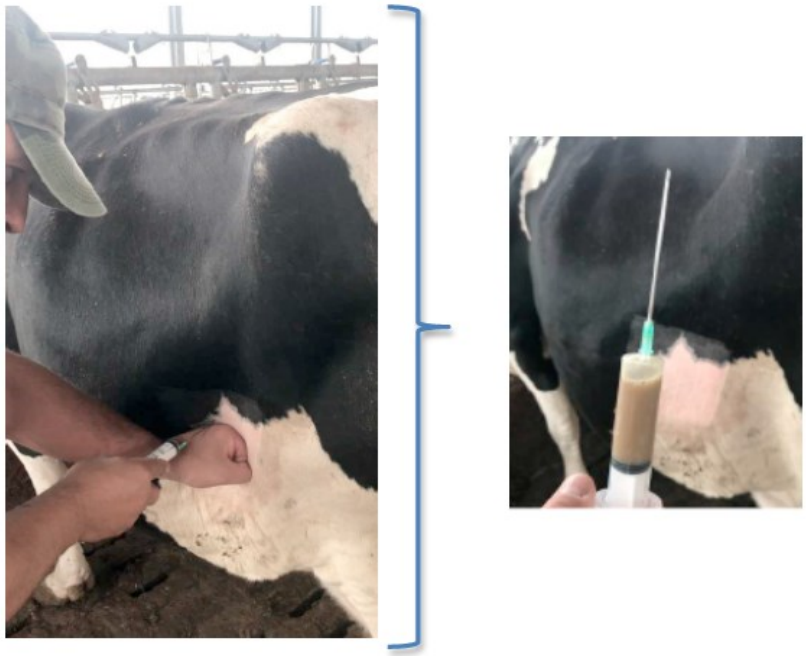


Figure 8 *– Ruminal fluid collection by rumenocentesis (Pictures kindly provided by Dr. Cristiano Barisani).*

With rumenocentesis the sampling point is on the left side of the animal, 15-20 cm caudo-ventrally to the costochondral junction of the last rib, at the level of the knee joint (Nordlund *et al.*, 1994) (Fig. 8). The identified area of about 20x20 cm should previously be shaved and disinfected.

In both cases the ruminal fluid collection should be carried out between 4 to 7 hours after feed administration, when the ruminal pH reaches the acidity peak (Morgante *et al*., 2007).

The preliminary data were obtained on rumen fluid samples from three fistulated Friesian heifers (5 mL/animal), fed a standard diet based on grass hay and 1 kg/d of a mixed concentrate fortified with minerals and vitamins. The animals were housed at the Agricultural Experiment Station in Piacenza (Italy), under the supervision of the Italian National Veterinary Services.

For the field survey, rumen fluid, blood and fecal samples were collected in 12 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 during at least a month before the collection and at sampling time. Only clinically healthy cows were included in the study.

Rumen fluids were collected by rumenocentesis (5 mL/animal), using a 13G (105 mm) needle (Vygon, Eocuen, France), about 5-6 hours after total mixed ration (TMR) feeding (as described by Trevisi *et al.*, 2014b), 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. From 20 of those animals rumen fluid was also collected by esophageal probe about 5-6 hours after total mixed ration (TMR) feeding.

Each sample of ruminal fluid was aliquoted in 3 fractions: a) for pH measurement (1 mL), b) for immunometabolic analysis (2 mL), and c) for immunological analysis (2 mL).

An aliquot of fluid (b) 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).

The other aliquot (c) was deep frozen after addition of dimethyl sulfoxide (DMSO) and fetal calf serum (FCS) (10% and 40%, respectively), for immunological and molecular assays.

Blood samples (9 mL/animal) were collected from the jugular vein in vacutainer tubes containing lithium heparin (Vacutainer; Becton Dickinson, Plymouth, UK), and immediately cooled in ice water (for inflammometabolic assays). 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 minutes 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.

Monensin Treatment

Forty-three healthy cows (13 heifers and 30 multiparous), hosted in an Experiment Station (Università Cattolica del Sacro Cuore, Piacenza Italy), were randomly allocated to two homogeneous groups: Monensin and Control. Monensin group cows received 0.6 mg/kg/die of Monensin by a Controlled Release Capsule (Kexxtone®, Elanco Animal Health, UK) 21 days before expected calving. The animals were frequently monitored during the transition period for health status, milk yield and quality, and inflammometabolic profile. Rumen samples were collected at 30 days in milk (DIM), 6 hours after feed distribution by rumenocentesis as previously described.

Ruminal pH measurement

In all cases the pH was measured immediately after sampling by a pH-meter (GLP 21, Crison Instruments SA, Alella, Barcelona, Spain).

Ruminal and fecal VFA measurement

The VFA concentration in rumen fluid and in the fecal extract was analysed by a gas chromatograph (model 7820A, Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-FFAP capillary column (30 m X 250 μm X 0.25 μm; Agilent J&W GC column) and a flame ionization detector. The oven temperature was 60°C held for 5 min and then increased by 5°C/min to 140°C. The injector temperature was 250°C and the detector temperature was 300°C. The injector was equipped with a glass liner of glass wool to separate particles of dirt from the sample. The samples were dosed by auto sampler at an injection size of 1 μL using the split method and a 25:1 splitting ratio. Hydrogen and air were used for flame ionization detection. The carrier gas was nitrogen, with constant flow of 1.78 mL/min, and pivalic acid was used as an internal standard (Ahmed *et al.,* 2013; Minuti *et al.,* 2014).

All the samples analysed in this study were processed in the lab within 4 hours after collection.

This study complied with Italian laws on animal experimentation and ethics enforced at that time (Legislative Decree 116/1992 enforcing EU Directive 86/609/EEC).

Processing of ruminal fluids and isolation of cells for flow cytometry and molecular assays

The frozen ruminal fluid samples 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 1687 x g, 20 minutes at 20°C. The harvested cells were diluted 1:2 with cold sterile PBS and placed on ice. Cells were then pelleted (441 x g, 10 minutes, 5°C), resuspended in 2 mL of PBS and centrifuged through 1 mL FCS layer (to separate mononuclear cells from bacterial cells) at 300 x g for 10 minutes at 5°C. The supernatant, containing bacteria, was discarded and the pellet, containing protozoa and leukocytes, was used for flow cytometry or RNA extraction.

RNA extraction from rumen wall samples

Total RNA was isolated from tissue (rumen wall) samples by the guanidine isothiocyanate method with minor modifications. Briefly, the samples were homogenized in 5 mL of guanidine isothiocyanate using a rotor-stator system (Ultra Turrax T25, Ika-Werke, Staufen, Germany). The lysate was centrifuged overnight at 42,000 rpm at 18ºC under a caesium chloride 5.7M layer

(Beckman ultracentrifuge Optima TL, Beckman Instruments, Inc., Palo Alto, CA, USA). The pellet was dissolved in sterile water and the RNA was precipitated with absolute ethanol and sodium acetate 3M pH 5.4 in dry ice for 2 h. After centrifugation in a microcentrifuge (1K15, Sigma, Osterode am Harz, Germany) at maximum speed for 30 minutes, the RNA pellet was dissolved in DEPC-treated water and stored at - 20 ºC.

The concentration of RNA was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm and 280 nm wavelength.

RNA extraction from ruminal fluid cells and saliva samples

Cell pellet obtained from saliva and ruminal fluids was lysed in 0.5 mL of TRIzol Reagent (Sigma–Aldrich, St. Louis, MO, USA) and then stored at -20ºC till use. Total RNA was extracted according to the manufacturer's instructions. Briefly, samples were thawed at room temperature for 5 minutes and 100 µl of chloroform were added at each sample. After a vigorous mix on vortex samples were centrifuged at 12,000 x g for 15 minutes at 4°C in order to extract the proteins to the organic phase, resolve DNA at the interface and deliver the RNA in the aqueous phase. The aqueous phase was transferred in a sterile 1,5 mL tube and 250 µl of isopropanol were added in order to precipitate the total RNA. After gentle inversions of the tube, samples were incubated at room temperature for 10 minutes and then centrifuged for 15 minutes, 12,000 x g at 4°C.

The supernatant was removed, the pellet was washed with 600 µl of 70% ethanol and finally the samples were centrifuged for 5 minutes, 7,500 x g at 4°C.

The supernatant was eliminated and total RNA was dissolved into 12 µl of DEPC-treated water.

The RNA concentration and quality (integrity) were then determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 and 280 nm wavelengths.

Primers design and DNA sequencing

Bovine mRNA and genomic sequences of target genes were obtained from GenBank and the accession numbers of all the sequences are listed in Table 1. Messenger sequences were aligned with genomic DNA sequences using the BLAST free software to identify exon/intron boundaries (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The primer pairs were designed using the Primer Express Software (Applied Biosystem, Foster City, CA, USA) on the messenger sequences of target genes and selected to produce amplicons spanning 2 exons. This way we were sure to amplify the cDNA and not the genomic DNA. Primers were purchased from Invitrogen (Carlsbad, CA, USA); their sequences are listed in Table 1.

These primer sets were then tested on cDNA extracted from rumen wall and sequenced for appropriate amplicon size and high-quality sequence.

Table I *– Primers sequences.*

Gene	Protein	Sequence	GI number	Amplicon
CD45	Bovine membrane tyrosine phosphatase	F:CTCGATGTTAAGCGAGAGGAAT R:TCTTCATCTTCCACGCAGTCTA	9944227	185 pb
IGLC	Immunoglobulin lambda constant	F: TCCTGGGTCAGCCCAAGTCC R: GGTGGTCTTCACGTTGCGG	56708413	167 pb
KRT5	Keratin 5	F:CAAGGTCCTGGACACCAAGT R:TCCAGCTGTCTCCTGAGGTT	56710316	113 pb
GAPDH *(housekeeping gene)*	Glyceraldehyde-3-phosphate dehydrogenase	F:GGCGTGAACCACGAAGTATAA R:CCCTCCACGATGCCAAAGT	89573946	116 pb

Qualitative PCR

Qualitative PCR was then used to verify the primers specificity. Each PCR reaction contained 5 ng of cDNA (from controlled samples of bovine tissues), 12.5µL of BioMix Red 2X with 50 mM $MgCl_2$ Solution (with a final concentration of 2.5 mM) (BIOLINE Reagents, London, UK), 0.3 µl (of 10 µM solution) of forward and reverse primers in a 25 µL reaction volume. PCR cycling parameters were: one cycle of 94°C for 15 minutes, 30 cycles of:

- 94°C for 1 minute;
- 44°C for 1 minute (for CD45 and KRT5), and 54°C for 1 minute (for IGLC);
- 72°C for 1 minute;

followed by one cycle of 72°C for 7 minutes.

Electrophoresis and samples purification

PCR products were electophoretically separated on agarose gel (1.8%) (GellyPhor LM Agarose, Euroclone S.p., Pero-Milano, ITA). The gel preparation was done by boiling the agarose powder in a TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Gel was stained by adding GelRed

(Biotium, California, USA) to the final concentration of 0.5 µg/ml. The gel polymerized in a tray at room temperature and the samples and markers were loaded into the wells. Samples were subjected to constant voltage of 80 volts for 30 minutes in TAE buffer.

On UV light lamp the visible gel bands were excised and purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). The procedure was carried out following the manufacturer's protocol. Briefly, excised DNA bands were placed in a 1.5 ml tube, and 10 µl Membrane Binding Solution were added for each 10mg of gel slice. The samples were Vortexed and incubated at 50–65°C until gel slice was completely dissolved.

An equal volume of Membrane Binding Solution was added to the sample and transferred to a SV Minicolumn.

After incubation at room temperature for 1 minute, the samples were centrifuged at 16,000 × g for 1 minute, washed with 700 µl Membrane Wash Solution, centrifuged and washed again with 500 µl Membrane Wash Solution. After a centrifugation at 16,000 × g for 5 minutes the collection tube was empty and the column assembly recentrifuged for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol.

Finally, the minicolumn was transferred to a clean 1.5ml microcentrifuge tube and the DNA was eluted in 50µl of Nuclease-Free Water by centrifugation.

The DNA concentration and quality were then determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 and 280 nm wavelengths.

Once quantified, 30 ng of the sample in 17 µl of final volume, containing primers (2 µl), sample and water (15 µl), were submitted to Sanger sequencing at an external service (Eurofins Genomics, Germany).

Reverse transcription and Real time PCR

Total RNA from each sample (saliva and ruminal fluid cells samples) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions using random primers.

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, in MicroAmp optical 96-well plates. Each plate contained duplicates of each sample cDNA (9 µl), 2× Power Sybr Green PCR Master Mix (12.5 µl) (Applied Biosystem, Foster City, CA, USA) and the concentration of the primers was established at 300 nM each (0.3 µl of 10 µM solution). Saliva and rumen fluid samples were analysed for gene expression of CD45 (Leukocyte Common Antigen), IGLC (immunoglobulin light

chain, lambda gene cluster; B cells) and KRT5 (keratin 5; rumen epithelial cells). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was investigated as housekeeping gene.

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), at the following thermal cycle conditions, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. 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: $AU=2^{-\Delta Ct}$, where ΔCt=Ct (target gene) – Ct (housekeeping gene), where Ct (cycle of threshold) values were the mean of two test replicates (Schmittgen & Livak, 2008). The obtained values were multiplied by 10,000 in order to obtain the Arbitrary Units (AU).

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), respectively (Maddox *et al*., 1985). Ruminal cells were reacted with PBS-FCS-A 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 was established on the basis of the threshold of non-specific fluorescence defined by the negative control.

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 (18,407 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 IgG 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). 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 curve created with serial dilutions of a bovine reference serum (Bethyl Laboratories, cat. RS10-10) with known concentrations of IgM, IgA and IgG antibody isotypes.

Inflammometabolic assays

Plasma samples were analysed 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).

Blood metabolites were analysed 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).

Commercial kits were used to measure NEFA and zinc (Wako, Chemicals GmbH, Neuss, Germany), and β-OH-butyric acid (BHBA, kit Ranbut, Randox Laboratories Limited, Crumlin, County Antrim, United Kingdom Randox, UK). NEFA were determined with a Trinder end point [Acyl coenzyme A oxidase (ACOD)/Peroxidase (POD)] assay, after the acylation of coenzyme A by NEFA contained in the sample. BHBA was measured with a kinetic UV method, based on the oxidation of D-3 hydroxybutyrate to acetoacetate by 3-Hydroxybutyrate dehydrogenase like previously reported (Calamari *et al*., 2016).

Ceruloplasmin was measured using the method described by Sunderman *et al*., 1970, adapted to ILAB 600 condition, and this method is based on measurement of p-phenylenediamine dihydrochloride oxidation by the oxidase activity of ceruloplasmin; the methodology used was an endpoint type with a wavelenght of 546 nm and a coefficient of variation (CV) of 3.48.

Haptoglobin was measured using the method described by Skinner *et al.*, 1991, also adapted to ILAB 600 condition. Briefly, this method is based on peroxidase activity of methaemoglobin-haptoglobin complex measured by the rate of oxidation of guaiacol (hydrogen donor) in presence of hydrogen peroxide (oxidizing substrate); in this case the methodology used was an endpoint type with a wavelenght of 450 nm and a CV of 13.54.

ROMs were measured by a commercial kit (Diacron International s.r.l., Grosseto, Italy).

Plasma PON activity was measured by adapting the method of Ferré *et al*., 2002 to the ILAB 600. Briefly, 8 μL of plasma added to 125 μL of ultrapure water and 125 μL of assay buffer were incubated at 37°C. The assay buffer was composed of glycine buffer (0.05 mM, pH 10.5) containing 1 mM of paraoxon-methyl (Sigma-Aldrich, Seelze, Germany), 1 mM of CaCl2 and without NaCl. The rate of hydrolysis of paraoxon to p-nitrophenol was measured by monitoring the increase in absorbance at 405 nm, using a molar extinction coefficient of 18,050 L x mol^{-1} x cm^{-1} as suggested by Feingold et al. (1998). The unit of PON activity (U/mL) is defined as 1 nmol of p-nitrophenol formed per minute under the assay conditions (Bionaz *et al*., 2007).

NO_3, NO_2, and NOx metabolites were measured using the Griess test according to Gilliam *et al.*, 1993, and Bouchard *et al.,* 1999. Briefly, 400 µl samples were mixed with 100 µl of nitrate reductase buffer (0.05 U of nitrate reductase, 250 µM NADPH, 25 µM flavine adenine dinucleotide, 160 mM potassium phosphate buffer, pH 7.5) and incubated at 37°C for 3 h. An equal volume of Griess reagent (1% sulfanilamide, 0.1% n-(1-naphtyl)-ethylenediamine, 2% phosphoric acid) was added and total nitrite was evaluated by reading optical density of samples at 540 nm. The NOx concentration was determined by comparison with an NO_2 standard curve (0 to 25 µM).

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 always possible to analyze all the parameters in the same samples: FACS, Real Time PCR and immunoglobulin concentration. In particular, from the 128 cows sampled, Real Time PCR was performed on 98 animals, flow cytometry analyses on 93 animals and immunoglobulin tests on 77 animals from the 12 farms. All the parameters were investigated in 37 animals.

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 among leukocyte populations (flow cytometry analysis) or between ruminal immunoglobulin concentrations versus all the other parameters (two quantitative parameters at a time).

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 α=0.05. Tendencies were declared with p-value< 0.1.

The differences between the two groups, Monensin and Control cows, were analyzed by ANOVA (SAS Inst. Inc., Cary, NC), considering the fixed effect of treatment and parity, with a p-value =0.05; tendencies were declared with p-value< 0.1.

The parameters that demonstrated a high significant difference with the two-tails rho test, were also analysed using the partial correlation test of the software SPSS (IBM) in order to study the association between two parameters while keeping a third parameter constant (ruminal pH) and to avoid potential confounders.

Results

Primers design and DNA Sequencing

Primers design

In order to draw specific primers for CD45, IGLC, KRT5, and GAPDH, we first searched for primers already validated in the literature for *Bos taurus* as well as sequences of these genes in databases.

We were able to find only primers already validated for GAPDH, and we used those published by Leutenegger *et al.*, 2000 (Table 1).

As for the other three genes, and as already described above in the material and methods section, we designed new pairs of primers. The designed primers were first validated in a qualitative PCR assay using cDNA from ruminal wall tissue. After the definition of the optimal PCR protocol in terms of primers concentration, annealing temperature, $MgCl_2$ concentration, we purified and sequenced the amplicon of the expected length for each primer pairs.

As we got our chromatogram files of each amplicon (CD45, KRT5, and IGLC) from the sequencing instrument, we aligned them to our reference sequences (downloaded from the NCBI set) (Table 1), using BLAST free software in order to verify the specificity of the amplification (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

IGLC

The sequence obtained for IGLC gene (Fig. 9) showed a 97% homology to the genes of IGLC of *Bos taurus* (Fig. 10).

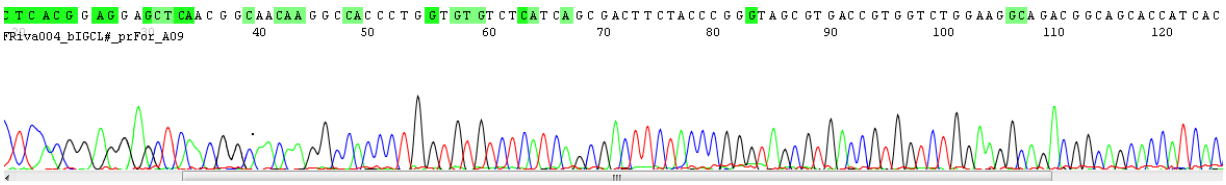


Figure 9 – *DNA sequencing result of the IGLC gene.*

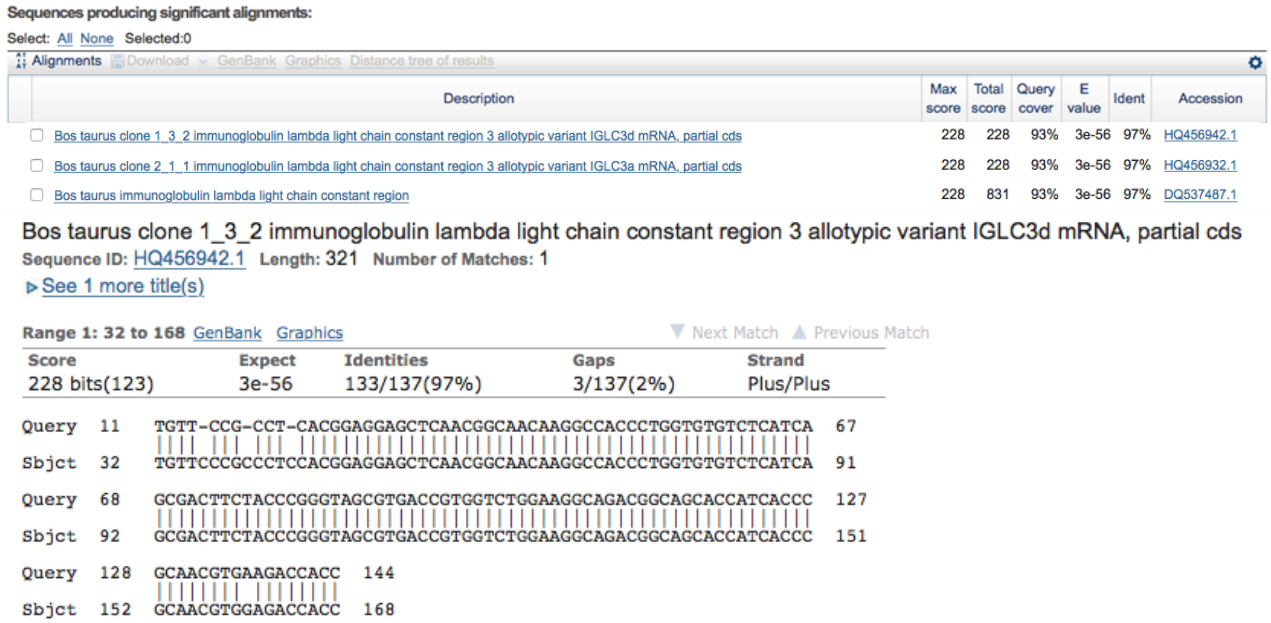


Figure 10 *– BLAST analysis of our sequenced DNA of IGLC gene, demonstrated that it is 97% homologue to the respective GeneBank sequence.*

CD45

The sequence of the amplicon obtained with CD45 primers (Fig. 11) had a 97% homology to the genes of CD45 of *Bos taurus* (Fig. 12).

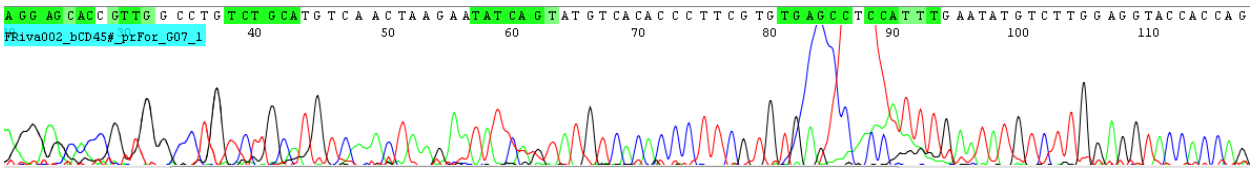
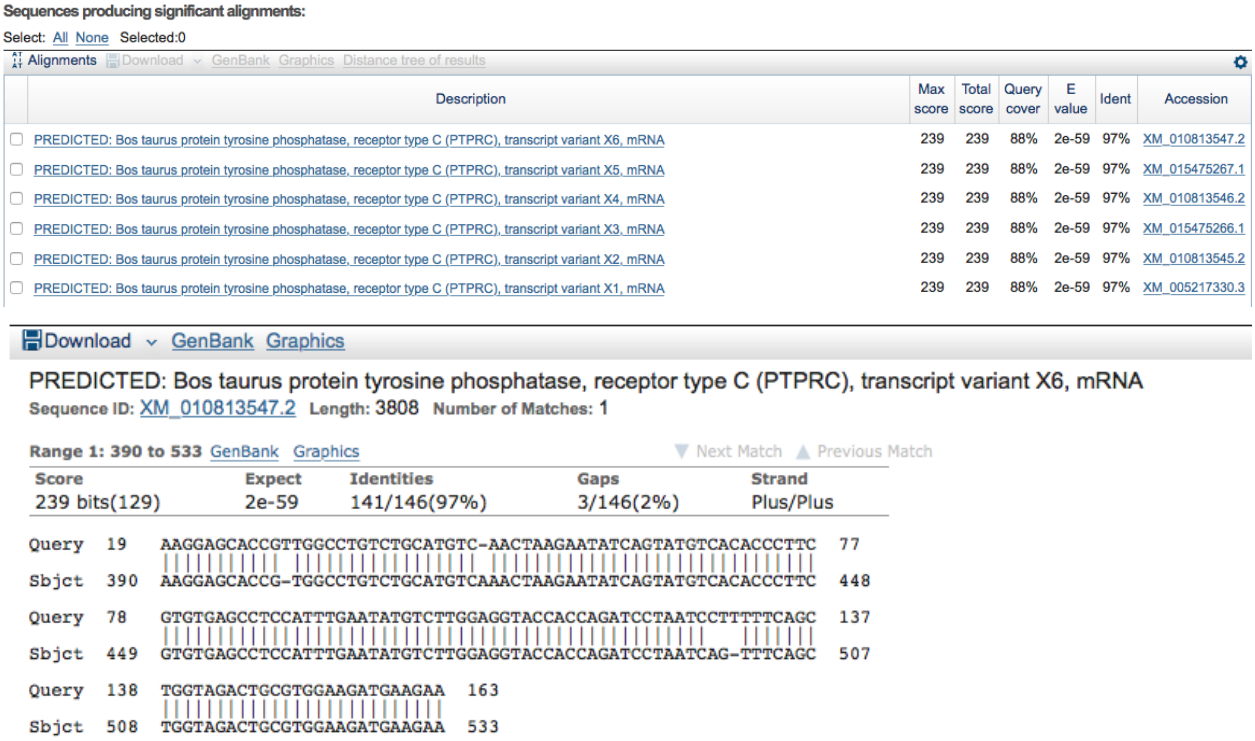


Figure 11 *– DNA sequencing result of the CD45 gene.*



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X6, mRNA	239	239	88%	2e-59	97%	XM_010813547.2
PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X5, mRNA	239	239	88%	2e-59	97%	XM_015475267.1
PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X4, mRNA	239	239	88%	2e-59	97%	XM_010813546.2
PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X3, mRNA	239	239	88%	2e-59	97%	XM_015475266.1
PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X2, mRNA	239	239	88%	2e-59	97%	XM_010813545.2
PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X1, mRNA	239	239	88%	2e-59	97%	XM_005217330.3

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PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type C (PTPRC), transcript variant X6, mRNA

Sequence ID: XM_010813547.2 Length: 3808 Number of Matches: 1

Range 1: 390 to 533 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
239 bits(129)	2e-59	141/146(97%)	3/146(2%)	Plus/Plus

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Query  19   AAGGAGCACCGTTGGCCTGTCTGCATGTC-AACTAAGAATATCAGTATGTCACACCCTTC  77
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Sbjct  449  GTGTGAGCCTCCATTTGAATATGTCTTGGAGGTACCACCAGATCCTAATCAG-TTTCAGC  507

Query  138  TGGTAGACTGCGTGGAAGATGAAGAA  163
            |||||||||||||||||||||||||||
Sbjct  508  TGGTAGACTGCGTGGAAGATGAAGAA  533
```

Figure 12 *– BLAST analysis of our sequenced DNA of CD45 gene demonstrated that it is 97% homologue to the respective GeneBank sequence.*

KRT5

The sequence of the amplicon obtained with KRT5 primers (Fig. 13) had a 93% homology to the genes of KRT5 of *Bos taurus* (Fig. 14).

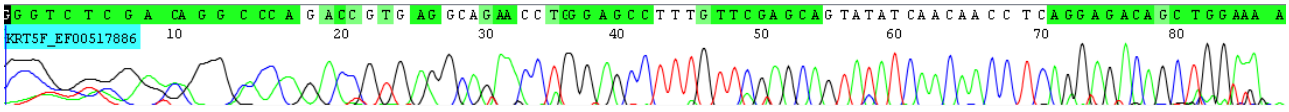
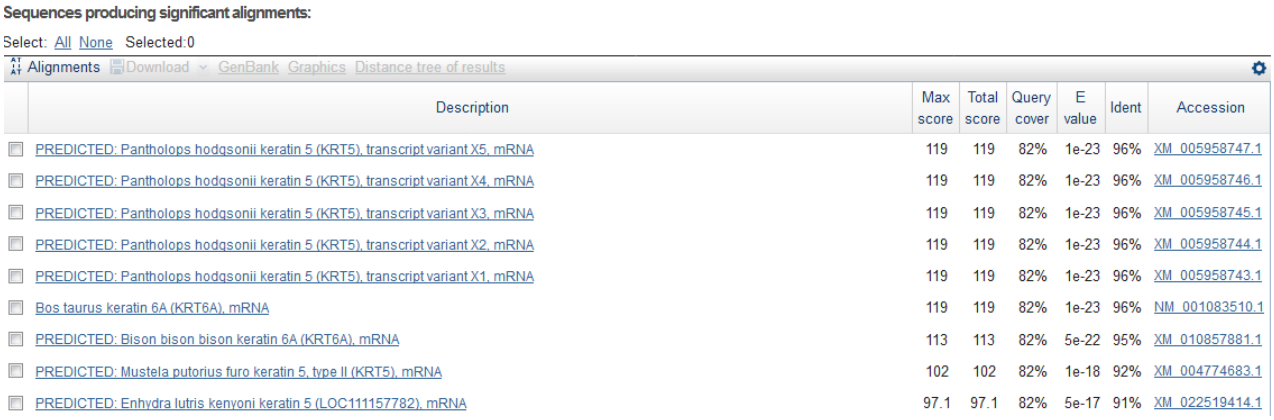


Figure 13 *- DNA sequencing result of the KRT5 gene.*



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Pantholops hodgsonii keratin 5 (KRT5), transcript variant X5, mRNA	119	119	82%	1e-23	96%	XM_005958747.1
PREDICTED: Pantholops hodgsonii keratin 5 (KRT5), transcript variant X4, mRNA	119	119	82%	1e-23	96%	XM_005958746.1
PREDICTED: Pantholops hodgsonii keratin 5 (KRT5), transcript variant X3, mRNA	119	119	82%	1e-23	96%	XM_005958745.1
PREDICTED: Pantholops hodgsonii keratin 5 (KRT5), transcript variant X2, mRNA	119	119	82%	1e-23	96%	XM_005958744.1
PREDICTED: Pantholops hodgsonii keratin 5 (KRT5), transcript variant X1, mRNA	119	119	82%	1e-23	96%	XM_005958743.1
Bos taurus keratin 6A (KRT6A), mRNA	119	119	82%	1e-23	96%	NM_001083510.1
PREDICTED: Bison bison bison keratin 6A (KRT6A), mRNA	113	113	82%	5e-22	95%	XM_010857881.1
PREDICTED: Mustela putorius furo keratin 5, type II (KRT5), mRNA	102	102	82%	1e-18	92%	XM_004774683.1
PREDICTED: Enhydra lutris kenyoni keratin 5 (LOC111157782), mRNA	97.1	97.1	82%	5e-17	91%	XM_022519414.1

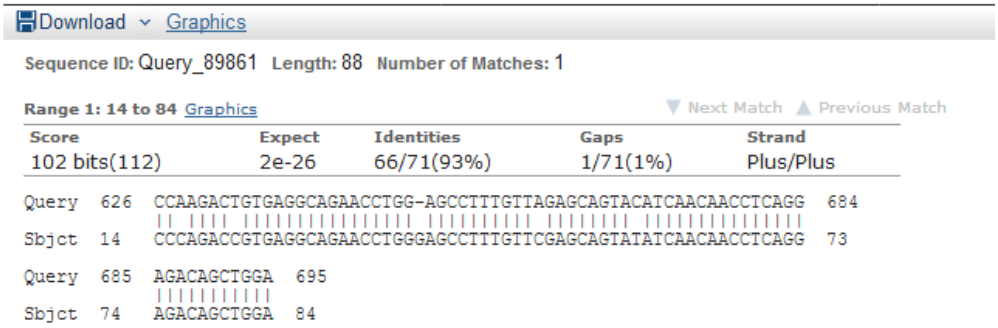


Figure 14 *– BLAST analysis of our sequenced DNA of KRT5 gene demonstrated that it is 93% homologue to the respective GeneBank sequence.*

In order to check the specificity of the primer pairs, representative amplicons from some rumen fluid and cell wall cDNAs were purified and sequenced. The sequences obtained were 93-97% homologue to the respective GeneBank sequences (Figures 10, 12 and 14). 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.

Identification and characterization of the rumen liquor cell subpopulations (preliminary results)

Previous studies demonstrated the presence of few leukocytes in the rumen fluid from cows among a great number of other cells (Trevisi *et al.*, 2009; Trevisi *et al.*, 2014a). The cells of the rumen liquor include more than 20 species of protozoa, at least six different species of fungi, and more than 300 different species of bacteria (e.g. Bacteroidites and Firmicutes), and a small number of leukocytes and epithelial cells. In order to isolate the leukocytes we edited a protocol previously described (Trevisi *et al.*, 2014a), that allowed us to remove the majority of bacteria, but not the protozoa from the rumen liquor. Moreover our protocol excludes also granulocytes.

We decided to characterize the composition of the rumen liquor leukocytes by flow cytometry analysis. The characterization was first set up on rumen liquor from three Frisian heifers with a surgical fistula of the rumen cavity housed at the Agricultural Experiment Station in Piacenza (Italy). We could confirm by flow cytometry analysis the presence of leukocytes at very low frequency, and also identify specific sub populations such as B cells (IL-A30$^+$), CD4$^+$ cells (IL-A12$^+$), CD8$^+$ cells (IL-A51$^+$), γδ T-cells (IL-A29$^+$), or monocytes (IL-A24$^+$) (Fig. 15). Those cells were detected with the same scatter characteristics of control bovine PBMC.

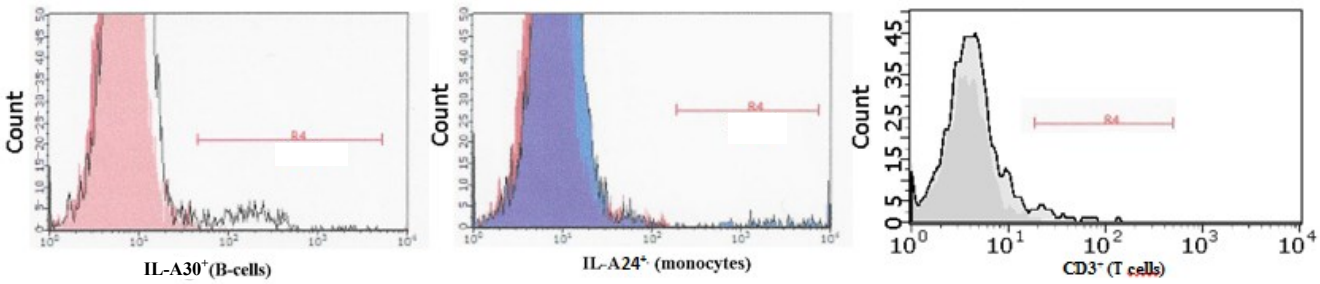


Figure 15 - *Non-bacterial mononuclear cells (NBMC) are selected by forward and side scatter, in order to define a viable region. The analysis of IL-A30$^+$, IL-A24$^+$ and CD3$^+$ cells is done on the viable gate, with respect to the negative control (overlapped histogram). The R4 region shows the percentage observed in 10,000 events.*

The presence of leukocytes was confirmed by qPCR by the detection of CD45 mRNA expression in the same rumen fluid samples (Fig. 16).

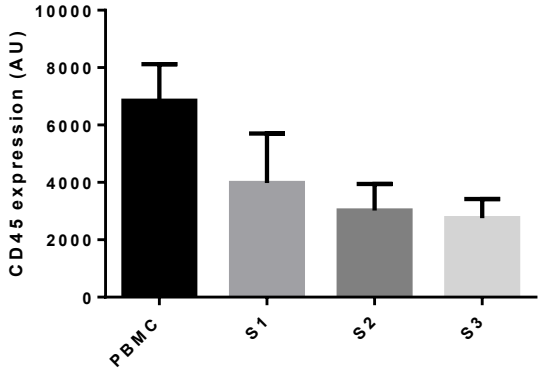


Figure 16 *– CD45 RT PCR comparison between control bovine PBMC and ruminal fluid samples from three fistulated cows (S1, S2 and S3).*

The sampling method slightly affects the rumen liquor pattern

Rumenocentesis is still not easily accepted among farmers as a method for sample collection, although it is considered the gold standard technique among the practitioners. So we investigated if the two different techniques of sampling (esophageal probe and rumenocentesis) could affect the composition of leukocyte subpopulation in rumen fluids. For that the ruminal liquor was collected from the same animals through the two different techniques; after the isolation of the ruminal cells we investigated by qPCR the expression of CD45. Both techniques allowed the detection of the leukocyte marker CD45 and in the majority of the samples (13/20) the two techniques demonstrated similar levels of expression (Fig. 17).

The differences underlined in some samples could be explained by the fact that rumen fluid, collected by the different methods, comes from different areas of the rumen (as previously described in bibliography), so the presence of leukocytes could be variable.

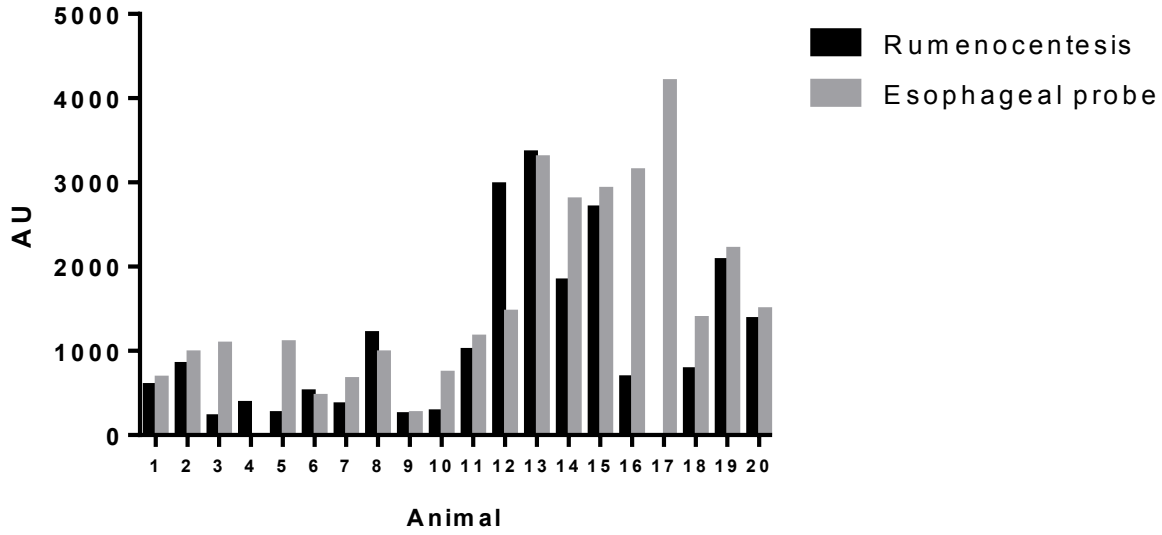


Figure 17 - *Expression pattern of CD45: rumenocentesis vs esophageal probe.*

Rumen leukocytes partly derive from saliva

In order to study the origin of rumen fluid leukocytes we collected saliva samples from 7 healthy cows. Cells were isolated from the saliva and analysed for the presence of leukocytes. Three samples were analysed by flow cytometry and four samples were analysed by Real Time PCR.

As shown in Figure 18A, the average number of total live cells was 6.9 x 10^3 cells/ml of saliva, with a range between 2.9 x 10^3 and 1.1 x 10^4 cells/mL. The average prevalence of $CD45^+$ (leukocytes) cells was 7.9%, corresponding to an average number of 4.28 x 10^2 cells/ml of saliva, with a range between 3.6 x 10^2 and 4.8 x 10^2 cells/mL (Table II).

All the samples analysed by qPCR expressed CD45 (leukocytes), KRT5 (epithelial cells) and IGLC (B cells) genes (Figure 18B), 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 (Figure 18B).

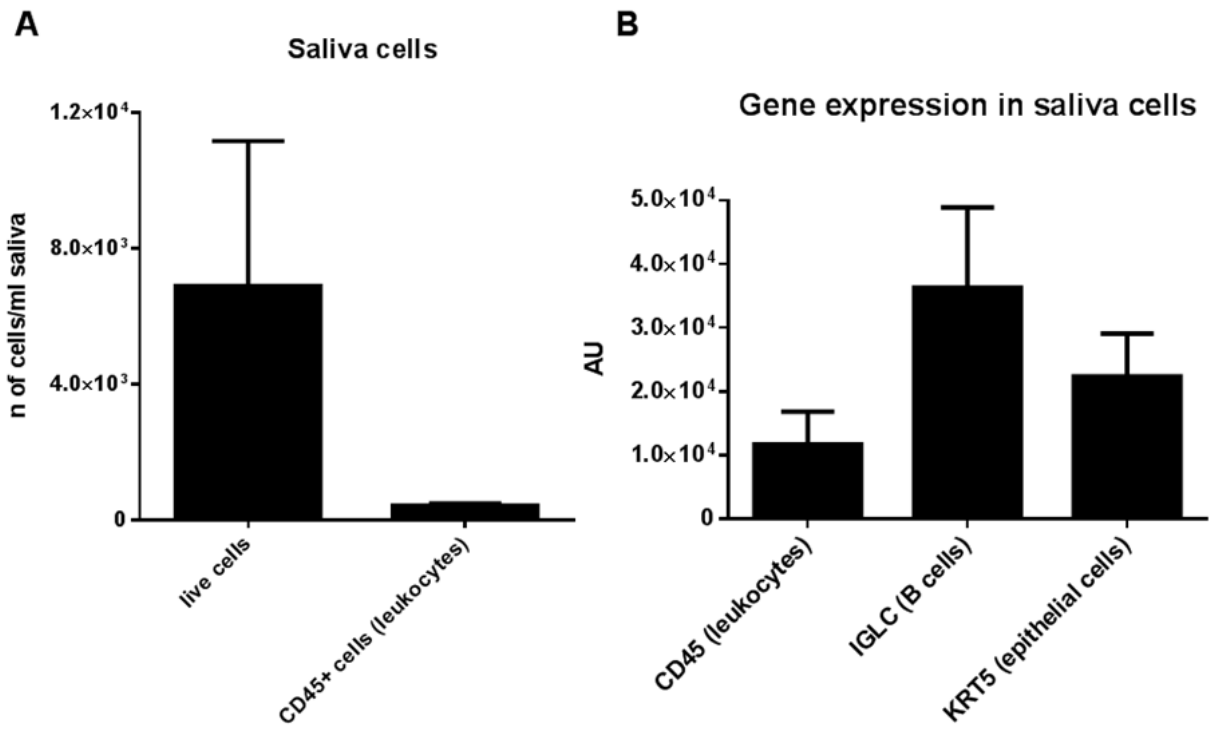


Figure 18 - *Presence of leukocytes in saliva samples.*

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 Ct x 10000). Results are expressed as mean ± 1 standard deviation in both panels.

Table II – *saliva samples results from flow cytometry.*

Saliva samples	Total number of cells/mL	% of live cells	Number of live cells	% of live $CD45^+$ cells	Number live $CD45^+$ cells
1	30800	9.4%	2895	12.4%	359
2	12780	50.33%	6432	7.5%	482
3	28600	39.8%	11383	3.9%	444
Average			6903	7.9%	428

The expression levels of CD45 and IGLC do not correlate to the number of cells, but only to the levels of expression of the two genes. Indeed in a single cell CD45 could be less expressed compared to IGLC. So our result only demonstrates the presence of cells expressing CD45 and IGLC, and the expression values cannot be directly compared.

Field survey

Preliminary results (Trevisi *et al.,* 2014a), obtained under experimental conditions, suggested that forestomachs play an important and active role in response to different stresses (e.g. diet modification, cytokines, microorganisms) and that ruminal fluid could be a new source of diagnostic information. In order to better understand the characteristics of the rumen liquor in the producing animals, we performed a field survey on 128 healthy animals from 12 different farms, analyzing a large number of parameters: hematological, metabolic, productive, and immunological. To this purpose, blood, milk, feces and rumen fluid were collected. The correlation among the different parameters was investigated with a statistical analysis. In this chapter we focus only on correlations with the highest level of significance, however all the correlation results (even those with low statistical significance) can be found at the end of this thesis in the supplementary results chapter.

CD45 gene expression in rumen fluid samples

CD45 gene expression (leukocytes) in ruminal samples was assessed in 98 animals from 11 farms by Real Time PCR, and it showed peculiar, farm-specific patterns of expression. As shown in figure 19, apparently the farms can be divided in two groups: farms with a compact pattern of CD45 gene expression, such as farm 7, 8, 9, 11 and 12 (with a low average value and standard deviation), and farms with a diffuse CD45 gene expression pattern among their animals, such as farm 2, 3, 4, 5 and 6 (with a high average value and Standard Deviation). 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 correlation between CD45 gene expression levels and ruminal pH ($p=0.0068$) (Table III).

CD45 expression was also positively correlated with the levels of total immunoglobulins ($p=0.0092$) in the rumen liquor (Table III).

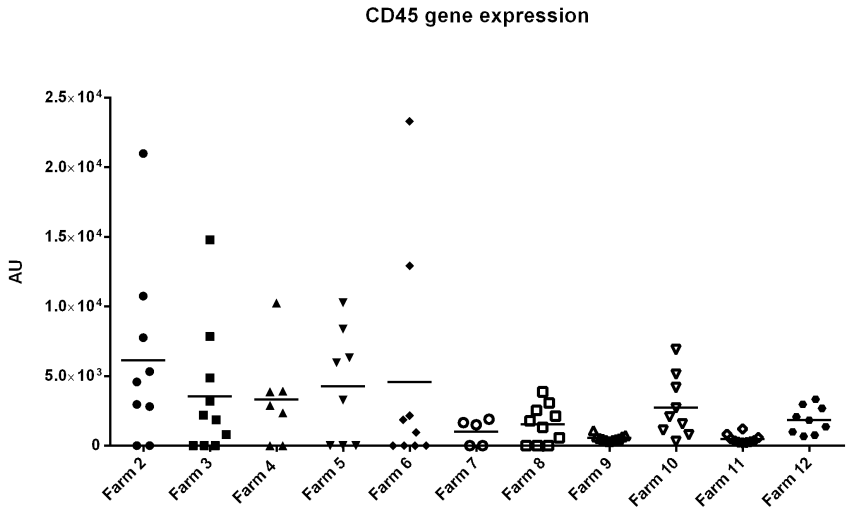


Figure 19 - *Field survey of CD45 mRNA expression in rumen fluid.*

CD45 mRNA expression was analysed 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 Arbitrary Units with the mean value (Delta Ct x 10000).

Table III - *Correlations between CD45 gene expression in rumen fluids, pH, Ig and prevalence of some leukocyte populations.*

	Parameter	Rho (Spearman)	Statistical significance	p-value	Sample source
CD45 gene expression (leukocytes)	Ruminal pH	0.27	**	0.0068	Rumen
	Total Ig in rumen fluid	0.36	**	0.0092	Rumen

The bivarial linear correlations were performed between CD45 gene expression and one parameter at a time.

B cells are the most frequent leukocyte population in rumen liquor

Rumen leukocytes were analysed by flow cytometry, and as in the preliminary results, different leukocyte populations at low concentrations were identified among the mononuclear, non-bacterial cells isolated from the rumen liquor of 93 animals from 12 farms. As shown in Figure 20, the most frequent mononuclear leukocyte population in 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 analysed 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 the isolation procedure.

The intra-assay coefficient of variation values amounted to 1.3, 0.4 and 1.6% for green fluorescence, FSC intensity and SSC intensity, respectively. The inter-assay coefficient of variation values amounted to 0.9, 0.3 and 2.6% for green fluorescence, FSC intensity and SSC intensity, respectively.

FACs analysis error is around 1-2%, so our estimated percentage below 2% of cells prevalence is obviously inaccurate. However, at the same time, the number of sampling carried out still allows the identification of B lymphocytes as the main leukocyte population in rumen fluid. The significance of this data derives from the statistical prevalence analysis (Chi-square or Fisher exact test) which compares the prevalence observed on the total number of analyzed events (10,000).

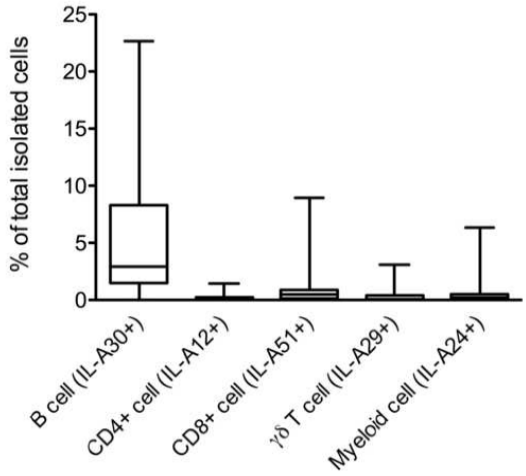


Figure 20 - *Leukocyte population profile in rumen fluid.*

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

Correlations among rumen liquor leukocyte profile, metabolic and hematologic parameters

In order to define new disease-predicting parameters in dairy cows, we searched any possible correlation between the leukocyte profile obtained by flow cytometry analyses and the other sets of parameters analysed. To this purpose, we included 93 animals from 12 farms.

Statistical analysis demonstrated a significant correlation between ruminal leukocyte populations, ruminal immunoglobulins and biochemical parameters. Interestingly, we observed a positive correlation between B cells (IL-A30) in rumen fluid and the pH of feces (Table IV - C). Moreover, the prevalence of IL-A29 and IL-A30-positive cells had a positive correlation with paraoxonase levels in plasma ($p < 0.0001$) (Table IV – B and C). Myeloid cells in the rumen liquor also showed a positive correlation, with bilirubinemia ($p = 0.0002$) (Table IV – E). The complete correlations analysis with the statistical significance between leukocytes and other parameters (including different leukocytes sub-populations) is recapitulated in table IV.

Table IV. *Correlations between rumen fluids different leukocytes populations, and metabolic and hematologic parameters.*

A	Parameter	Rho (Spearman)	Statistical significance	p-value	Sample source
ILA12 ($CD4^+$ cells)	ILA29 (gamma delta T cells)	0.48	****	<0.0001	Rumen
	ILA24 (myeloid cells)	0.50	****	<0.0001	Rumen
	Glutamic oxaloacetic transaminase (GOT)	-0.21	*	0.04	Plasma

The bivarial linear correlations were performed between ILA12 expression and one parameter at a time.

B	Parameter	Rho (Spearman)	Statistical significance	p-value	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 were performed between ILA29 expression and one parameter at a time.

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

	Calcium (Ca)	-0.34	**	0.0012	Plasma

The bivarial linear correlations were performed between ILA30 expression and one parameter at a time.

D	Parameter	Rho (Spearman)	Statistical significance	p-value	Sample sources
ILA51 ($CD8^+$ cells)	IgMCD21 (mature B cells)	0.43	***	0.0002	Plasma

The bivarial linear correlations were performed between ILA51 expression and one parameter at a time.

E	Parameter	Rho (Spearman)	Statistical significance	p-value	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 were performed between ILA24 expression and one parameter at a time.

Correlations among rumen liquor immunoglobulins and metabolic parameters

Given that B cells are the major population among rumen 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 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) (Table V). IgM, but mainly total Ig presented a strong correlation with some blood ion concentrations (K^+, Na^+) (Tables V-VI). Also, Ig showed a strong positive correlation with urea in blood (p<0.0001), a parameter that might reflect dry matter intake, provided that intake and degradability of crude proteins are analogous (Table VI).

Table V *– Correlations between IgM concentration in rumen fluid and biochemical or hematological parameters.*

	Parameter	Rho (Spearman)	Statistical significance	p-value
IgM in rumen fluid	Ruminal pH	0.32	**	0.0055
	Ruminal valeric acid	-0.45	****	<0.0001
	Ruminal volatile fatty acids	-0.41	***	0.0004
	$ME^{¥}$ (Mature	-0.52	***	0.001

	equivalent milk production)			
	Na	0.47	****	<0.0001
	Total Ig in rumen fluid	0.60	****	<0.0001

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

Table VI *– Correlations between total immunoglobulins concentration in rumen fluid and biochemical or hematological parameters.*

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

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. The inter- and intra-assay coefficients of variation were 3.76 and 1.13% for ROM, 4.63 and 0.46% for NO_2, and 6.46 and 2.32% for NOx. NO_3 has been calculated by difference from NOx and NO_2.

Note: Other (less significant) results can be found in supplementary tables at the end of this thesis.

Correlations among metabolic and hematologic parameters

Even if the main goal of this thesis was to find correlations between immunological parameters and already consolidated diagnostic parameters (metabolic and hematologic), we also verified the existence of correlations among metabolic and hematologic parameters underlying some already proved associations, like for example the fact that ruminal pH and VFA production are negatively correlated (Table VII – A), or that a decrease in albumin concentration in blood serum goes along with an increase of positive APPs (e.g. haptoglobin, globulins) (Table VII – B). Also the positive correlation between zinc and cholesterol concentrations in blood serum (Table VII – G) has been observed before (Koo *et al*., 1981; Saiki *et al*., 2009).

Similarly, the positive correlation between NEFA and ROMs in serum (Table VII – F) agrees with previous studies in cattle (Bernabucci *et al.,* 2005).

Other correlations, like the positive one between PON and albumin (Table VII – D), are in agreement with previous works (Bionaz *et al*., 2007; Trevisi *et al*., 2012b).

Table VII *- Correlations among metabolic and hematologic parameters.*

A	Parameter	Rho (Spearman)	Statistical significance	p-value
Ruminal pH	Ruminal volatile fatty acids	-0.60	****	<0.0001
	Ruminal acetic acid	-0.42	****	<0.0001
	Ruminal propionic acid	-0.59	****	<0.0001
	Ruminal valeric acid	-0.41	****	<0.0001

B	Parameter	Rho (Spearman)	Statistical significance	p-value
Albumin (serum)	Calcium (serum)	0.50	****	<0.0001
	Creatinine (Serum)	0.35	****	<0.0001
	Globulin (serum)	-0.48	****	<0.0001
	Haptoglobin (serum)	-0.31	****	<0.0001
	Paraoxonase	0.34	****	<0.0001

C	Parameter	Rho (Spearman)	Statistical significance	p-value
Urea (serum)	Glutamic oxaloacetic transaminase (GOT) (serum)	0.43	****	<0.0001
	Percentage cell volume (PCV) (blood)	0.37	****	<0.0001
	Linfocytes (blood)	0.33	****	<0.0001
	Eritrocytes (blood)	0.42	****	<0.0001
	Paraoxonase (Serum)	-0.33	****	<0.0001

D	Parameter	Rho (Spearman)	Statistical significance	p-value
Paraoxonase (PON) (serum)	NEFA (serum)	0.33	****	<0.0001
	Urea (serum)	-0.33	****	<0.0001
	Albumin (serum)	0.34	****	<0.0001

E	Parameter	Rho (Spearman)	Statistical significance	p-value
BHBA (serum)	Glucose (serum)	-0.50	****	<0.0001
	Ruminal butyric acid	0.40	****	<0.0001
	Ruminal propionic acid	-0.43	****	<0.0001

F	Parameter	Rho (Spearman)	Statistical significance	p-value
NEFA (serum)	Feces pH	-0.33	****	<0.0001
	ROM (serum)	0.40	****	<0.0001
	No_x (serum)	0.33	****	<0.0001

G	Parameter	Rho (Spearman)	Statistical significance	p-value
Zinc (serum)	Cholesterol (serum)	0.35	****	<0.0001

Treatment with Monensin affects different metabolic and immunological parameters in the rumen

Considering the positive metabolic effects that Monensin seems to offer as rumen bolus during the transition period, we investigated its effects on rumen fermentations and innate immune responses of the forestomachs. In practice, we decided to introduce a programmed alteration of rumen fermentations. The purpose of this intervention was to verify if and to what extent the innate immune system reacts to an objective change in ruminal metabolism. In this context, the use of Monensin should be seen as a model for the study of innate immune responses to non-infectious stressors.

Table VIII - *Metabolic and immunological data.*

	Unity	Control (medium value)	Monensin (medium value)	p-value
pH		6.39	6.51	NS
ammonia	mg/L	93.3	58.7	<0.05
Propionic acid	mol/ 100 mol	25.9	28.4	<0.1
Acetic acid/propionic acid	ratio	2.8	2.5	<0.1
B lymphocytes	% of total cells	2.5	1.66	<0.01
IgM	ng/mL	54.3	34.1	<0.1
Total Ig	ng/mL	7243.3	2076.9	<0.1

Treatment with Monensin led to significant modifications of some ruminal and hematological parameters. Monensin-treated cows showed a similar ruminal pH (around 6.45), but lower concentration of serum ammonia (58.7 vs 93.3 mg/L of Ctrl; $p < 0.05$), and a higher concentration of ruminal propionic acid (28.4 vs 25.9 mol/100 mol of Ctrl; $p < 0.10$, tendency) when compared with control animals (Table VIII). Treated animals also showed a reduced acetic:propionic ratio (2.5 vs 2.8 of Ctrl; $p < 0.10$, tendency) (Table VIII). Moreover, Monensin vs Control cows showed a lower number of B lymphocytes (1.66 vs 2.50%; $p < 0.01$), T lymphocytes (not significant) and a lower amount of immunoglobulins (not significant) (Table VIII).

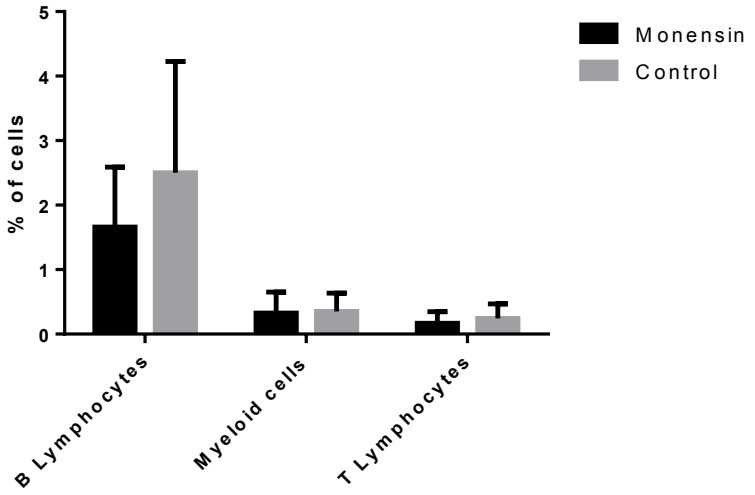


Figure 21 – *Percentage of infiltrating leukocytes in the rumen of treated animals (Monensin) versus control animals.*

The reduced prevalence of T lymphocytes was more marked in heifers than pluriparous cows after Monensin treatment ($p < 0.10$, tendency, vs Control).

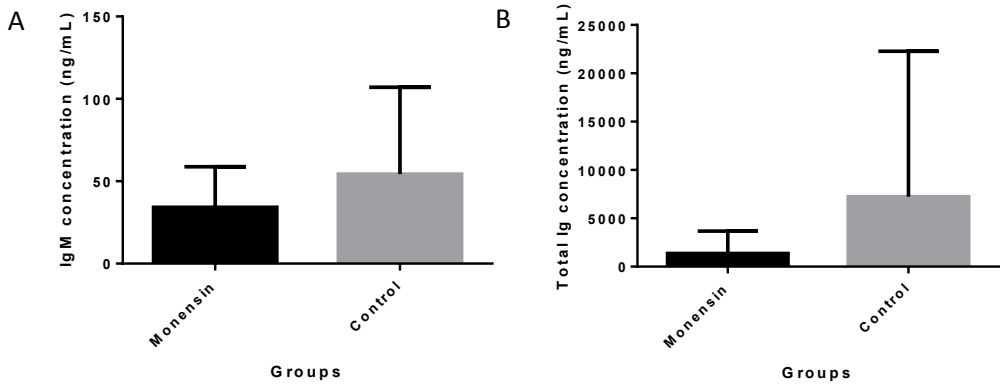


Figure 22 *– A) Ruminal fluid IgM concentration; B) Ruminal fluid total Ig concentration.*

Treatment with Monensin also influenced the concentration of immunoglobulins in ruminal fluid samples; indeed we demonstrated a reduction of both total Ig and IgM in treated cows (Fig. 22).

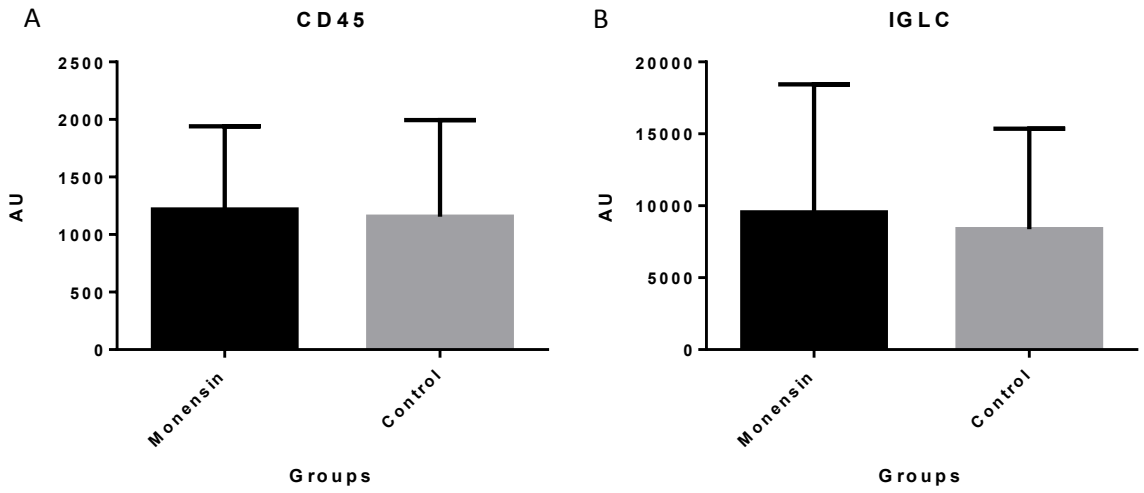


Figure 23 *– A) CD45 mRNA expression in rumen fluid; B) IGLC mRNA expression in rumen fluid.*

At a molecular level we didn't find any significant statistical difference between the two groups (Fig. 23).

Preliminary data on a diagnostic kit set up for rumen liquor immune profile

The data presented above confirm that nutritional and physiological changes in dairy cows can modify the innate immune response of forestomachs and that the evaluation of the rumen fluid can help to value animal health and welfare.

The rumen can control the fermentative activity by the innate immune system, which can recognize "danger" signals and implement containment strategies against the stressors, mediated by signals transmitted to the lymphoid tissue of the oral cavity. In order to standardize the analysis of the immunological pattern of the rumen fluid (a useful approach in addition to the classic diagnostic protocols), we developed a diagnostic kit for molecular, immuno-phenotypic and metabolic parameters on rumen liquor.

This kit is based on the association between metabolic stress and innate immune response, and we expect that it could provide useful data toward the interpretation of complex, multifactorial

pathologies; it may also provide useful indications on diets in use for ruminants, but of course the findings should be accompanied by a careful evaluation of clinical, zootechnical, and management data.

The kit should be applied to at least 10 animals for herd/group following rumenocentesis within 4 to 7 hours after administration of feed.

The single ruminal liquid sample should be distributed immediately and aliquoted in 3 fractions: a) for pH measurement, b) for immunometabolic analysis, and c) for immunological analysis: an aliquot of 1 mL of rumen fluid in the 1st test tube (a), at least 2 mL (up to 4 mL) in the 2nd one (b), and finally 2 mL in the 3rd tube, which already contains 2 mL of dimethyl sulfoxide (DMSO) and fetal calf serum (FCS) (10% and 40%, respectively), for immunological and molecular assays. Then, all the tubes should be immediately transferred into an ice bath and kept until the delivery to the lab (this should be done within 2 hours after the collection).

On the basis of this scheme, we tested the efficacy of this protocol on two groups of 6 (animals without clinical symptoms) and 4 (animals with clinical symptoms of rumen stasis) respectively, with similar profiles, representing specific homogeneous parts of the herd.

Table IX – *IgM and total immunoglobulins concentration in rumen fluid samples.*

Animal	Group	Total Ig (ng/ml)	IgM (ng/ml)	IgM/Total Ig ratio
1	Healthy	> 10.000	> 720	Not calculable
2	Healthy	> 10.000	> 720	Not calculable
3	Healthy	787	81	0,10
4	Healthy	2405	548	0,23
5	Healthy	1932	324	0,17
6	Healthy	4336	203	0,05
7	Ruminal stasis	1189	188	0,16
8	Ruminal stasis	> 10.000	> 720	Not calculable
9	Ruminal stasis	6824	325	0,05
10	Ruminal stasis	1170	163	0,14

The ruminal fluid samples presented distinct and diffuse quantitative leukocyte infiltration and immunoglobulin (Ig) alterations.

In some cases there is an abnormal accumulation of Ig and IgM/total Ig ratio > 0.1 (Table IX). IgMs accumulation could be related to recent responses to infectious or noninfectious stressors, thereby indicating a recent deviation from physiological conditions.

In general, an interferon-gamma response was not detected, but we could identify a disturbance of homeostasis characterized by abnormal levels of infiltrating T lymphocytes and, in some cases, monocyte/macrophage cells. In some samples, we could also detect infiltration values > 3% of B

lymphocytes, which are the major leukocyte population in rumen fluid. For all the three leukocyte populations detected a higher infiltration percentage was noticed on animals suffering of ruminal stasis when comparing with healthy ones (Fig. 24).

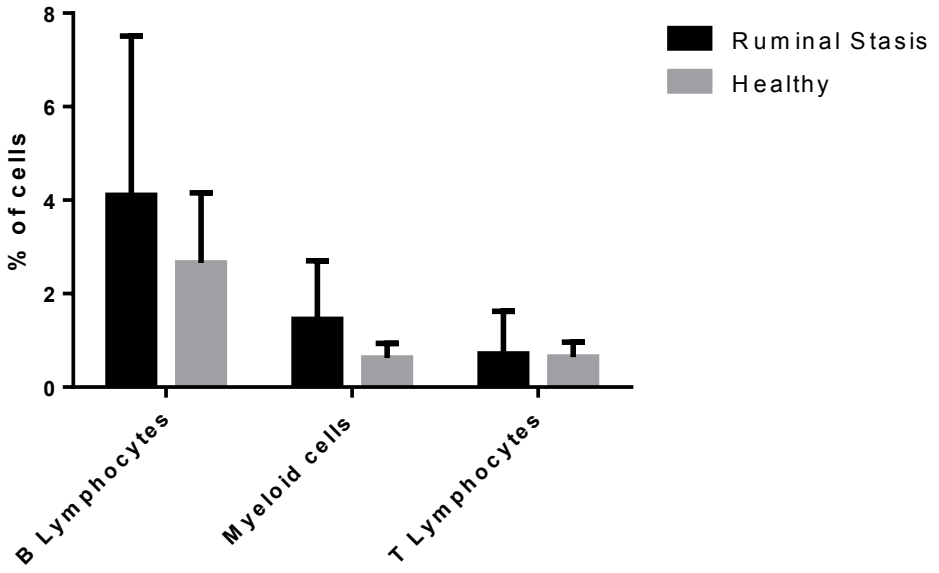


Figure 24 *– Percentage of infiltrating leukocytes in the rumen.*

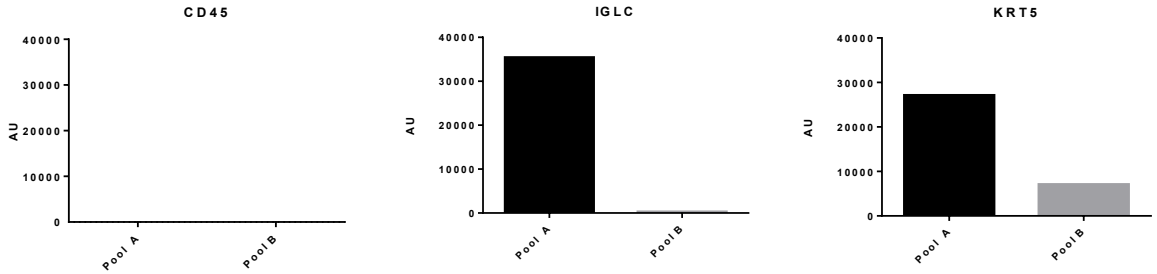


Figure 25 - *Pool A: bovine without clinical symptoms. Pool B: bovine with clinical symptoms of rumen stasis.*

For the qPCR assay, 2 pools of samples were set up. Pool A with 6 samples from cows without clinical symptoms, while pool B with 4 samples from cows showing signs of rumen stasis (Fig. 25). CD45 analysis failed in both pools probably due to the sensitivity level of the assay. In the group with pathology there was a marked reduction in IGLC gene expression. In addition to that, pool B showed a lower concentration of epithelial wall cells (KRT5 gene), which may indicate a change in the turnover rate of such cells.

Discussion

The main function of the ruminal and reticulum mucosa of the forestomachs is that of absorbing metabolites from bacterial digestion, which accounts for the role of forestomachs in digestive problems.

Recently, a possible active role of the forestomachs in the response to alimentary disorders, inflammatory and infectious processes arising both in the gastro-intestinal tract and elsewhere has started some interest inside the scientific community. This role of forestomachs should be set into a correct conceptual framework, which highlights the very functions and goals of the innate immune response.

The immune response represents an ancestral and complex system of reactions to damages that disturb the homeostatic balance of the host. Accordingly, the innate immune system can develop an inflammatory response to achieve a better ability to deal with different stressors. At the same time, this response needs to be accurately controlled to avoid tissue damage and excessive waste of metabolic energy (Janeway *et al.,* 2001, Abbas *et al.*, 2016).

In the homeostatic state, healthy subjects are constantly exposed to inflammatory stimuli of different nature (e.g. LPS, nucleic acids, NEFA, etc.) at very low concentrations, and this condition determines a constant alert of the innate immune system. When these stimuli exceed certain values (concentration and/or duration), a state of irreversible inflammatory response may start (Amadori, 2016). In high-yielding dairy cattle, these phenomena are unfortunately common, and usually accompanied by a general condition of poor immune competence of the individuals (Trevisi *et al*., 2014). This feature should be viewed in a broader scenario. The achievement of high production levels in dairy farms, seen during the last years, has been associated to increasing difficulties of these animals to adapt to the environment. This translates into increased use of veterinary drugs, a higher risk of metabolic and infectious diseases and a reduction of life expectancy as well as reduced fertility (Oltenacu *et al*., 2010; Amadori, 2016).

In this conceptual framework, previous studies had demonstrated the functional capacity of bovine forestomachs to start innate immune responses to abnormal diet profiles, including expression of both stimulatory and inhibitory receptors of the innate immune system in the epithelial cells of forestomachs, and modulation of cytokines and leukocyte infiltrations in rumen fluids through a continuous cross-talk between forestomachs and oral lymphoid tissues (Trevisi *et al*., 2009; Trevisi *et al*., 2014a). This could mean that innate immune responses can be initiated,

sustained and expanded in bovine forestomachs by an integrated system including receptors, signaling molecules, cytokines and infiltrating leukocytes.

The project of my PhD thesis has confirmed some of those findings in a field scenario and set further important milestones.

The presence of infiltrating leukocytes in rumen fluids was confirmed by flow cytometry analysis and Real Time PCR. Indeed, we were able to identify different populations of leukocytes infiltrating the ruminal fluid.

In order to better characterize the cell population composition of the rumen liquor, we investigated by Real Time PCR the expression of CD45 gene as marker of total leukocytes, IGLC as marker of B cells (that preliminary results of flow cytometry analysis revealed as the prevalent type of leukocytes present in rumen fluid samples) and KRT5 as marker of squamous epithelial cells in the rumen liquor.

CD45 (CD stands for cluster of differentiation) also known as PTPRC (Protein tyrosine phosphatase receptor type C) gene, was originally called leukocyte common antigen (LCA), and has been chosen because it is expressed in all cells of hematopoietic origin except red cells, and can be used as a marker of leukocytes (Tizard *et al.*, 2013).

The immunoglobulin light chain has an important role in maintaining antibody conformation, antigenic moiety recognition, and immunoglobulin repertoire diversification. Two distinct immunoglobulin light chain isotypes have been described in mammals, kappa and lambda, and these are expressed in different ratios in the different animal species (Chen *et al*., 2008).

In domestic animals such as cattle the knowledge about immunoglobulin light chain is still limited (Aitken *et al*., 1999; Zhao *et al*., 2006), but it is known that the light chain repertoires in these animals are dominated by the lambda chains (Griebel *et al*., 1994; Butler, 1997), while kappa chains are expressed at a very low level (Ford *et al*., 1994; Arun *et al*., 1996).

Each lambda (λ) light chain is coded by three clusters of genes, and these are called IGLV, IGLJ, and IGLC. The IGLC genes code for the constant region (Fc) of the chain and are expressed exclusively by B cells (B Cell Receptor) (Tizard *et al*., 2013), making these genes perfect candidate as B lymphocytes markers.

The KRT5 gene provides instructions for making a protein called keratin 5. Keratin 5 is expressed primarily in basal keratinocytes in the epidermis, specifically in the stratified epithelium lining the skin and digestive tract, including the ruminants' forestomachs (Ramirez *et al*., 1994; Atkinson *et al*., 2011).

The specific expression of KRT5 by epithelial cells made it a perfect candidate as forestomach epithelial cell marker, and this enabled us to compare leukocyte and epithelial cell quantity modifications in each rumen fluid sample.

GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as reference gene for quantitative analysis of gene expression. The use of at least one housekeeping gene is essential in an extremely sensitive method like RT-qPCR, since data normalization helps the correction of experimental variations (e.g. differences in the amount of initial material or sampling loading) (VanGilder *et al.*, 2008; Nestorov *et al*., 2013). In order to be considered a good reference gene this must display constitutive and stable expression in all cell types/tissues and treatment regimens under consideration, and also be expressed at similar levels as the genes of interest (in our case CD45, IGLC and KRT5).

GAPDH is a metabolism-related gene, and together with actin-β is one of the most common reference genes used in bibliography. In a recent review of 128 works, it has been highlighted that three genes - GAPDH, actin-β and 18S rRNA - were used in 72% of those studies, as single normalizing genes (Chapman *et al*., 2015). GAPDH has been suggested as a good reference gene in different studies (Goossens *et al.,* 2005; Robinson *et al.*, 2007). Recently, scientific community opened a debate on the use of a single or multiple reference genes and the choice of the best reference gene. Some authors support the use of GAPDH as reference gene (Radonic *et al.*, 2004; Lisowski *et al.*, 2008). We decided to use GAPDH as a single reference gene because it is well characterized in cattle and we had to use a single reference gene for technical reasons (small amount of total RNA).

On the whole, our results indicate that cattle show different levels of leukocyte infiltration into rumen fluids, a common event down-regulated during ruminal acidosis (low pH), and affected by both local and systemic inflammatory conditions.

When comparing rumenocentesis with esophageal probe sampling, we isolated leukocytes with both techniques, and in the majority of the cases with similar results. However, whenever some differences seem to occur, this is probably due the fact that samples collected with different techniques derive from different areas of the rumen that could present a different composition.

Even if rumenocentesis is often not well accepted by farmers, it is preferable because it leads to the most accurate results (no contamination of the sample with saliva occurs), and that means that pH values do not change due to the sampling technique. Comparative studies showed that the pH of samples taken with esophageal probe or with rumenocentesis can differ by values ranging from 0.28 to 1.1 pH units (Enemark, 2008). When performed by an expert practitioner, rumenocentesis is

faster (less stress for the animals), and the sample is for sure collected from the rumen ventral sac (the ideal anatomical site for the collection). Importantly, rumen liquor sampling is an easy manual task for a trained veterinary practitioner, which does not cause any harmful side effect to the animals. In our study, hundreds of samplings were performed in the field and not even one side effect was observed in the animals under study. This was also reported in other studies (Nordlund *et al.*, 1994; Nordlund *et al.,* 1995; Garrett *et al*., 1999; Kleen *et al*., 2004; Bramley *et al*., 2008).

Besides confirming the presence of a small number of leukocytes in the rumen liquor, we further characterized the subpopulations of leukocytes present in this matrix.

By flow cytometry analysis we identify myeloid, $CD4^+$, $CD8^+$, gamma delta T cells, and B cells. However we cannot exclude the presence of granulocyte polymorphonuclear cells, because our isolation protocol does not include them.

We found a possible origin for the cells populations in rumen liquid, and it seems that at least some of those cells originate in the oral cavity and arrive in the forestomachs within saliva during the deglutition process. Studies in human medicine point to an origin of leukocytes in the oral cavity from the gingival crevice (the space between the surface of the tooth and the free gingiva); among the infiltrating leukocytes, neutrophils are the most common; these produce α-defensins, endued with anti-bacterial and anti-viral functions (Schiött *et al.*, 1970, Darnell *et al*., 2006; Dawes *et al*., 2015).

The detection of leukocytes in the saliva is a very interesting result considering the volume of saliva that every day reaches the rumen cavity in adult dairy cows (±200-250 L/day). Because of the particular digestion of ruminants, these cells can travel back and forth during the rumination between the oral cavity and forestomachs, enabling a continuous exchange of information, and potentially orchestrating an immune and inflammatory response to specific stressors. This hypothesis could explain how the rumen can react to systemic pathological conditions, and vice versa, how pathological conditions of the rumen can affect the immune response in other districts.

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 real 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).

Our field study outlined previously unreported relationships between productive, metabolic and immune parameters, which could have an important impact on diagnosis and characterization of production diseases and digestive troubles. 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. Our data agree with the results of a meta-analyses of SARA experiments (Zebeli *et al.*, 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. Similar results were observed by Stefanska *et al.*, 2018, that verified an increased production of SAA (considered by Gozho *et al*., 2005 the most sensitive APP responding to inflammatory stimuli) in SARA-positive animals.

In agreement with our working hypothesis, our results showed a correlation between inflammatory markers (e.g. paraoxonase, a negative acute phase protein) in blood, and some markers in the rumen liquor (e.g. gamma delta T cells, and B cells). 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.

On the whole, our results confirm and expand the scope of previous studies 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. A negative correlation between rumen pH levels and VFA concentrations

was also recognized in other recent study (Stefanska *et al.*, 2018). Another interesting result is the negative correlation between calcium concentration in plasma and the number of B cells in the rumen liquor; Eckel *et al.*, 2016 hypothesized that calcium inhibits LPS to bind to plasma high-density lipoproteins (HDL), and so hypocalcemia could be a protective response to LPS. In Hypocalcemia condition, the absorbed LPS could bind to HDL easily, and neutralized and eliminated without activating the immune system. Also Stefanska *et al.,* 2018 demonstrated a negative correlation between HDL and LPS receptors (CD14 and TLR4) expressed by blood leukocytes and a positive correlation between calcium and the same receptors. 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 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 has 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 *et al.*, 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 potassium (K) and sodium (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 correlation data with urea levels in this study, and with previous studies (Trevisi *et al*. 2007; Trevisi *et al*. 2010).

When comparing metabolic and hematologic parameters we also found some interesting correlations. For example, a positive correlation between zinc and cholesterol concentrations in blood serum was observed, demonstrating a close association between capture of zinc in liver

during inflammatory events and reduction of the high-density lipoproteins synthesis (Bertoni *et al.*, 2013). In opposite, the effect of nutritional status of zinc on lipoproteins content is a more controversial subject.

Also the positive correlation between NEFA and ROMs in serum agrees with previous works in cattle (Bernabucci *et al.*, 2005) and suggests the increase of oxidative stress during periods of high lipid mobilization. Finally a strong positive correlation between PON and albumin is in agreement with previous studies (Trevisi *et al*., 2012b; Bionaz *et al*., 2007), and this support the idea that PON behaves as a negative APP in cattle.

For the statistical analysis, we decided to perform bivariate correlations because we aimed to understand which parameters could influence others or be mutually correlated, but for such a complex analysis with more than 80 parameters under study, we found some weaknesses; in particular, some parameters had many values equal to zero (more than 10% of the samples). In order to confirm the stronger correlations we also made an analysis of the partial correlations to exclude the influence of an external parameter.

In order to investigate the capability of the forestomachs immune system to react to a specific and standardized metabolic change, we set up the Monensin model. Monensin is a widely used molecule in ruminant animal feeds (Łowicki *et al*. 2013; Butaye *et al*., 2003), because it increases the production of rumen propionic acid and fosters liver gluconeogenesis (Matsuoka *et al*., 1996). The treatment led to a lower concentration of ammonia and a higher concentration of propionic acid (with a reduced acetic:propionic ratio). Treated animals also showed a lower infiltration of B and T lymphocytes and lower concentrations of immunoglobulins (IgM and total Ig), that could be directly associated to the decrease of B cells present in the same samples.

The activity of the immune system was more pronounced in the rumen of control cows, suggesting that Monensin could have contributed to stabilize the rumen milieu and to attenuate the inflammatory responses that commonly occur in forestomachs around calving. These data were supported by clinical inspections with a significant lower incidence of diseases observed in the first two months of lactation in Monensin-treated cows. This was in line with metabolic data showing significantly lower NEFA and BHBA concentrations in treated cows after calving.

Finally, a diagnostic kit was tested in two groups of animals: one of healthy subjects and one of ruminal stasis suffering animals. We detected differences between the two groups in terms of leukocytes infiltration (flow cytometry analysis) and Ig presence (ELISA). At a molecular level the group with overt pathology showed a marked reduction in IGLC and KRT5 gene expression.

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), in association with inflammo-metabolic blood parameters. In this scenario, further experiments are badly needed to characterize the immunological profile of the rumen liquor under different and specific pathological conditions.

Some of the described markers in this thesis could represent an important adjunct to established laboratory procedures. In particular, they could 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.

These new results can actually open a “new road” to a correct understanding of production diseases of high-yielding dairy cows. This means that immunological analyses in veterinary sciences could be used as a complement to clinical, behavioral, genetic and/or zootechnical studies by adding fundamental and interesting new information. Most important, immunology could actually be the foundation of disease risk assessment procedures for improved herd control and management, providing early diagnostic data and allowing, this way, more effective therapeutic treatments of cattle at risk.

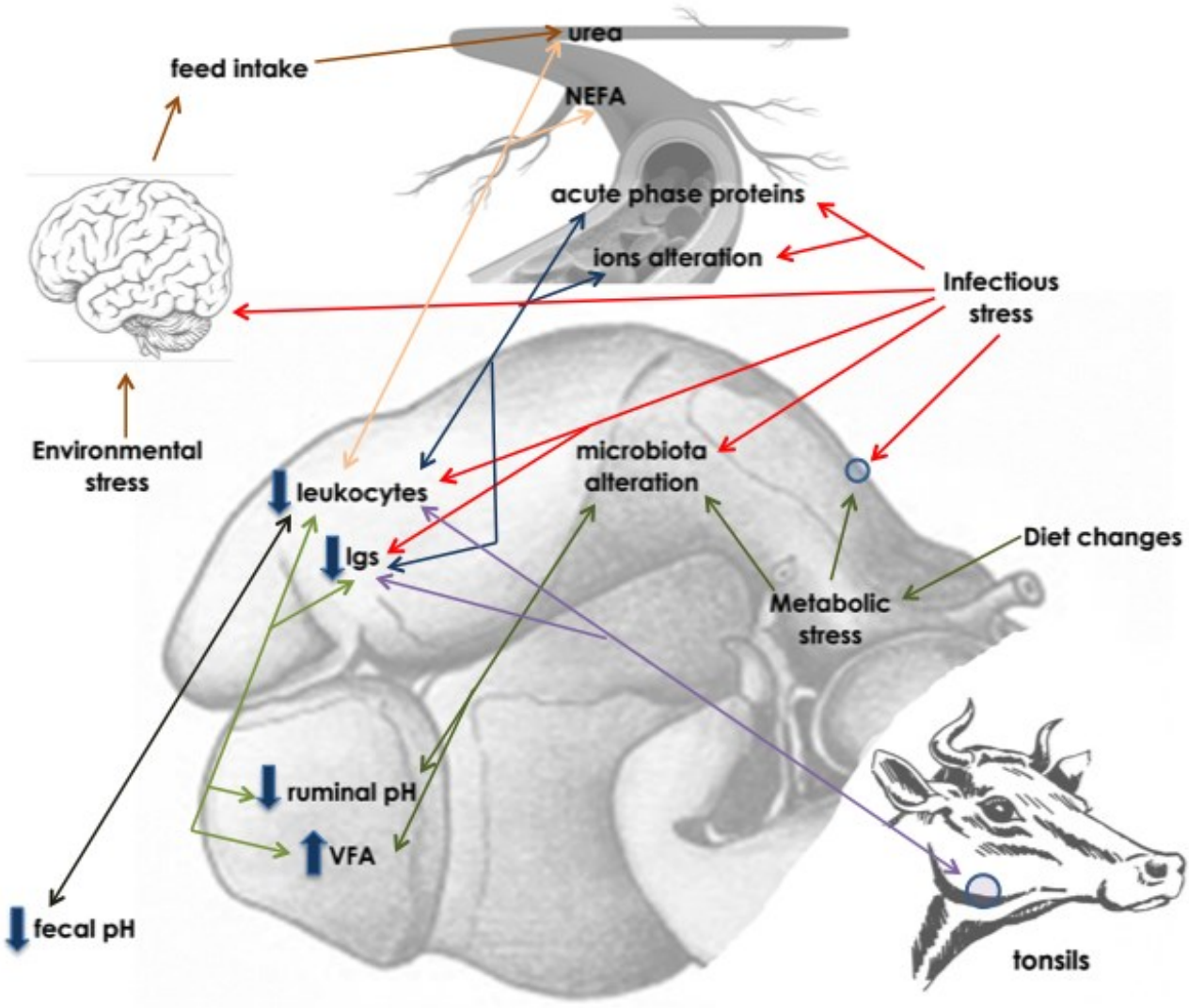


Figure 26 - Summary scheme of the correlation among different stressors and the rumen responses. *Different stresses can affect directly or indirectly the rumen activity. For example infectious stresses (e.g. LPS, TLRs ligands), can alter the rumen microflora, stimulate the ruminal wall trough the TLRs or influence the CNS. Moreover during the peripartum period the cow diet change (starch consumption increase in order to face the energy requirements for the milk production) can lead to a metabolic stress with a possible alteration of the ruminal microflora with an increase production of VFA (altered propionate:acetate ratio), a decrease of the rumen pH, and the production of metabolites able to stimulate the immune response of the rumen wall. The alteration of the microflora is also induced by Monensin treatment in order to improve the production of propionate. Finally the environmental stress (e.g. poor hygiene, social disturbances) influences the CNS that promotes defense responses such as the reduction of the feed intake. The results of my thesis demonstrated a positive correlation between the ruminal pH and the leukocytes infiltration and the amount of immunoglobulins (Igs). The increased production of VFA can lead to a higher absorption of NEFA in the blood; that are known to inhibit the functionality of the leukocytes. In my thesis a negative correlation between the NEFA concentration in blood and the leukocyte infiltration in the rumen was observed. Systemic stress can activate the acute phase response causing an alteration of the inflammo-metabolic profile in the serum that is correlated with the immunologic profile of the rumen liquor (some of the serum acute phase proteins, APPs, are correlated with leukocyte infiltration and the levels of Igs in the rumen fluid). Stress may also alter the ion concentrations in the serum and this is also correlated with leukocyte infiltration and the levels of Igs in the rumen fluid. Finally, the presence of leukocytes in the rumen fluid, partly derived from saliva, can sustain, during the rumination, a continuous cross talk with the oral cavity lymphoid tissue (such as the tonsils) for subsequent responses and effector activities.*

In conclusion, this scheme (that summarizes the results of my thesis and the previous results obtained by Trevisi et al., 2014) underlies different relations among ruminal pH, blood inflammo-metabolic profile and the immune parameters of rumen fluid, suggesting a possible role for the immunologic rumen profile analysis in the diagnosis of subclinical pathological conditions.

Blue circle represents the rumen wall that is able to express innate proinflammatory (e.g. TLR4, caspase-1, IL1β) and inhibitory (e.g. IL1-R8, IL-10) molecules; purple circle represents the tonsils (which represent a critical link between ruminal bolus and lymphoid tissue of the oral cavity); while arrows represent correlations between different parameters.

Conclusions and Future Plans

My PhD thesis represents a preliminary study that needs further investigations in order to better understand the real origin of the leukocytes in the rumen liquor, to characterize the immune profile changes of the rumen liquor during specific pathological conditions and to deeply validate our diagnostic kit.

Further studies could be also helpful to understand when the changes of the rumen fluid immune profile represent a cause and when it is an effect observed after exposure to whatever stressor.

Our survey put the basis for further investigations, and our future plan is to study the modifications of the immunological pattern of the rumen fluid in specific metabolic or non-metabolic pathological conditions (e.g. laminitis, mastitis, acidosis). At the moment we still don't know if these immune parameters modifications are a cause or a consequence; indeed the response to a stressor (infectious or not) is very complex and aims to contain the noxae, but when the response is too strong or too long, can cause itself damage to the host.

In the future, we aim to look for any possible correlation between rumen liquor profiles and specific production diseases (metabolic and infectious). Accordingly, if any strong correlation should be evidenced, we will set up new diagnostic tools for the determination of rumen liquor profile to be used on farm.

Validating our diagnostic kit and defining immunologic/molecular profiles linked to certain pathologies could be very helpful to the diagnosis and early treatment of different pathologies. This could be based on the critical association between metabolic stress and innate immune response, thus, useful data could be collected for the interpretation of complex, multi-factorial pathologies. The kit could also provide useful indications concerning diets for ruminants, along with a careful evaluation of clinical, zootechnical and management data.

Supplementary data

The tables of all the significant correlation results there were not included in the thesis are included as supplementary material.

Table I-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
CD45 gene expression (leukocytes)	Butyric acid	-0.24	*	0.025
	Igs	-0.42	**	0.0044
	Milk liters	-0.21	*	0.036
	ILA29 (gamma delta T cells)	-0.29	*	0.024
	IL-A24	-0.27	*	0.042

Table II-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
ILA12 (CD4+ cells)	Mean corpuscular volume (MCV)	0.26	*	0.012
	K	0.27	**	0.0098
	Paraoxonase	0.34	**	0.0011
	ILA51 (CD8+ cells)	0.23	*	0.028

Table III-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
ILA29 (gamma delta T cells)	IG	0.48	**	0.0041
	Mean corpuscular volume (MCV)	0.32	**	0.0028
	Mean Corpuscolar Hemoglobin	0.27	*	0.124
	P	-0.24	*	0.029
	Mg	0.33	**	0.0023
	Beta-hydroxybutyrate (BHBA)	-0.25	*	0.022
	Nitric oxyde total metabolites (NOx)	-0.22	*	0.039
	NO_2	-0.24	*	0.027
	MHC I	0.22	*	0.039
	ILA30 (B cells)	-0.28	**	0.0091
	IgMCD21 (mature B cells)	0.27	*	0.024
	CD45 gene expression (leukocytes)	-0.29	*	0.024

Table IV-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
ILA30 (B cells)	Ruminal urea	-0.22	*	0.048
	Acetic acid (%)	0.23	*	0.038
	Hematocrit	-0.31	**	0.0033
	Mean corpuscular hemoglobin concentration	0.30	**	0.0041
	Eosinophils number	0.29	**	0.0065
	Eosinophils (%)	0.25	*	0.016
	Glycemia	-0.25	*	0.016
	Mg	-0.25	*	0.016
	Na	0.22	*	0.038
	K	-0.23	*	0.026
	Albumin	-0.21	*	0.042
	Creatinine	-0.23	*	0.028
	Non esterified fatty acids (NEFA)	-0.24	*	0.022
	Total reactive oxygen metabolites (ROMt)	-0.26	*	0.012
	NO2	-0.25	*	0.016
	NO3	0.21	*	0.044
	ILA29 (gamma delta T cells)	-0.28	**	0.0091
	IgMCD21 (mature B cells)	0.36	**	0.002
	IgM in rumen fluid	0.27	*	0.045
	Total Ig in rumen fluid	0.30	*	0.020

Table V-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
ILA51 (CD8+ cells)	Mean corpuscular hemoglobin concentration	-0.27	*	0.010
	Red cell distribution width	0.30	**	0.0039
	Platelet number	-0.21	*	0.043
	Cholesterol	-0.29	**	0.006
	Bilirubin	0.31	**	0.003
	ILA12 (CD4+ cells)	0.23	*	0.012
	ILA46	0.33	**	0.015

Table VI-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
ILA24 (myeloid cells)	Butyric acid	-0.27	*	0.018
	ME[§] (Mature equivalent milk production)	-0.28	*	0.039
	IG	0.36	*	0.041
	Erythrocyte number	-0.24	*	0.024
	Mean corpuscular hemoglobin concentration	-0.24	*	0.022
	Percentage cell volume (PCV)	-0.26	*	0.014
	urea	-0.27	*	0.011

	Paraoxonase	0.26	*	0.013
	Glutamic oxaloacetic transaminase (GOT)	-0.22	*	0.039
	ILA51 (CD8+ cells)	0.33	**	0.015
	CD45 gene expression (leukocytes)	-0.27	*	0.042

Table VII-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
IgMCD21 (mature B cells)	Ruminal urea	-0.26	*	0.033
	Acetic acid	-0.25	*	0.037
	Butyric acdi	-0.35	**	0.004
	Isobutyric acid	-0.25	*	0.042
	ME[§] (Mature equivalent milk production)	-0.34	*	0.045
	Somatic cell count (SCC)	-0.31	**	0.0089
	Body condition score (BCS)	-0.25	*	0.032
	Red cell distribution width	0.32	**	0.0073
	Bilirubin	0.29	*	0.013
	ILA29 (gamma delta T cells)	0.27	*	0.024
	ILA30 (B cells)	0.36	**	0.002
	ILA51 (CD8+ cells)	0.43	***	0.0002
	ILA24	0.44	***	0.0001

Table VIII-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
IgM in ruminal fluid	Acetic acid	-0.34	**	0.004
	Propionic acid	-0.35	**	0.0035
	Butyric acid	-0.24	*	0.049
	Isobutyric acid	-0.25	*	0.037
	Veleric acid (%)	-0.37	**	0.0019
	Isovaleric acid	-0.25	*	0.036
	Fecal alcaline phosphatase	-0.25	*	0.035
	Mean corpuscular volume (MCV)	-0.30	**	0.0087
	Mean corpuscular hemoglobin concentration	0.35	**	0.0024
	Glycemia	-0.23	*	0.049
	Ca	-036	**	0.0015
	K	-0.37	**	0.0011
	Cl	0.24	*	0.039
	ILA30 (B cells)	0.27	*	0.045
	Total Ig in rumen fluid	0.60	****	<0.0001

Table IX-S

	Parameter	Rho (Spearman)	Statistical significance	p-value
Total Ig in rumen fluid	Acetic acid (%)	0.25	*	0.036
	Propionic acid	-0.27	*	0.022
	Isobutyric acid	-0.25	*	0.033
	Valeric acid	-0.26	*	0.030
	Isovaleric acid	-0.31	**	0.0088
	Volatile fatty acids	-0.30	*	0.011
	Fecal pH	0.29	*	0.012
	ME[§] (Mature equivalent milk production)	-0.44	**	0.0071
	Mean corpuscular volume (MCV)	-0.35	**	0.0021
	Mean corpuscular hemoglobin concentration	0.37	**	0.0013
	Neutrophils/Limphocytes ratio	-0.23	*	0.042
	Eosinophil number	0.30	*	0.010
	Neutrophil (%)	-0.24	*	0.035
	Eosinophil (%)	0.26	*	0.025
	Ca	-0.36	**	0.0018
	globulines	0.31	**	0.0078
	Albumin/globulin ratio	-0.34	**	0.0026
	Creatinine	-0.29	*	0.013
	Non esterified fatty acids (NEFA)	-0.31	**	0.007
	Total reactive oxygen metabolites (ROMt)	-0.25	*	0.03
	NO2	-0.26	*	0.026
	ILA30 (B cells)	0.31	*	0.020
	CD45 gene expression (leukocytes)	0.36	**	0.0092

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