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DIAGNOSIS AND PREVENTION OF SUBACUTE RUMINAL ACIDOSIS IN DAIRY CATTLE

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Riassunto

L’acidosi ruminale è un disturbo metabolico-fermentativo presente nell’allevamento della vacca da latte e nel bovino da carne. Attualmente la definizione di acidosi si basa sul pH ruminale e può manifestarsi in forma acuta, cronica e subclinica (sub-acuta). Al fine di ottenere un aumento delle produzioni di latte, le vacche ad alta produzione sono alimentate con diete altamente energetiche e a base di cereali che a seguito della rapida fermentazione ruminale dell’amido e degli zuccheri inducono un accumulo di acidi grassi volatili e/o lattato, che associato a una ridotta capacità tampone a livello ruminale causa una caduta del pH. L’acidosi ruminale subacuta (SARA) è caratterizzata da un recupero spontaneo di ripetuti picchi di basso pH ruminale, assente o transitorio accumulo di lattato ruminale e lievi sintomi clinici durante i cali di pH ruminale. SARA causa ingenti danni economici sia diretti che indiretti nell’allevamento della vacca da latte a causa della diminuita qualità e quantità di latte prodotto, delle maggiori spese veterinarie, della prematura macellazione dell’animale. I sintomi clinici di SARA nel singolo animale sono subdoli, ritardati di settimane o mesi dopo l’evento acidotico, non patognomonici e comuni ad altre numerose patologie. Il pH ruminale inoltre varia considerevolmente a seconda di vari fattori quali: tempo intercorso dopo il pasto, momento del giorno, punto di campionamento all’interno del rumine, tecniche di campionamento utilizzate per raccogliere il liquido ruminale. Per queste ragioni e per i problemi nell’ottenere campioni di liquido ruminale rappresentativi, SARA risulta essere molto spesso sottovalutata. Allo stato attuale, per diagnosticare SARA si misura il pH ruminale utilizzando varie tecniche: alcune di esse sono considerate invasive (fistolizzazione ruminale), o moderatamente invasive (ruminocentesi), forniscono rilievi puntiformi del pH ruminale, perdendo quindi

sensibilità nella diagnosi di acidosi ruminale (ruminocentesi, sonda orogastrica) o possono portare a contaminazioni con la saliva che altera il pH del campione (sonda orogastrica). Per ovviare a tali inconvenienti con questa tesi si è cercato di individuare metodi diagnostici (diretti ed indiretti) poco invasivi, rapidi, economici e di facile esecuzione per rilevare la presenza e definire la gravità dell'acidosi ruminale (capitoli 3 e 4). Si è inoltre indagata l'efficacia di diversi metodi di prevenzione (capitoli 5, 6, 7 e 8).

Il primo contributo sperimentale (Effect of induced ruminal acidosis on blood variables in heifers; capitolo 3) è stato eseguito utilizzando un quadrato latino 3 × 3 (3 diete × 3 periodi). A sei manze non gravide (incrocio Valdostana x Blu Belga) è stata somministrata una delle tre diete contenenti differenti livelli di amido (% sostanza secca): basso (17.3%) come controllo (CT), medio (MS) per indurre SARA (33.4%) e alto (HS) per indurre l'acidosi ruminale acuta (42.8%). Gli animali sono stati alimentati tre volte al giorno (ore 08:00, 12:00 e 18:00). Il pH ruminale è stato misurato di continuo ogni 10 minuti tramite boli, che hanno la caratteristica di fluttuare all'interno del liquido ruminale, e tramite ruminocentesi eseguita 4 ore dopo la distribuzione della razione al 4° giorno di ogni periodo sperimentale. Il coefficiente di regressione tra i due metodi (boli vs. ruminocentesi) è risultato pari a 0.56 (P = 0.040). La somministrazione di CT, MS e HS ha determinato delle differenze nel tempo speso sotto le soglie di pH 5.8, 5.5 e 5.0 e in diverse variabili compresa l'ingestione (7.7 vs. 6.9 vs. 5.1 kg/d per CT, MS e HS, rispettivamente; P = 0.002), pH ruminale nadir (5.69 vs. 5.47 vs. 5.44; P = 0.042), pH ruminale medio (6.50 vs. 6.34 vs. 6.31; P = 0.012), livello di emoglobina (11.1 vs. 10.9 vs. 11.4 g/dL; P = 0.010), conta piastrinica (506 vs. 481 vs. 601 K/µL; P = 0.008), HCO_3^- (31.8 vs. 31.3 vs. 30.6 mmol/L; P = 0.071) e proteina legante i lipopolisaccaridi (LBP) (5.9 vs. 9.5 vs. 10.5 µg/mL; P < 0.001). Applicando un'analisi discriminante canonica

multifattoriale (CDA), cinque variabili plasmatiche (emoglobina, volume piastrinico medio, β-idrossibutirrato, glucosio ed emoglobina ridotta) sono state in grado di discriminare significativamente lo stato ruminale fisiologico: SARA o acidosi ruminale acuta (Wilks' $\lambda = 0.282$, F approx = 3.76, df1 =15, df2 = 97, $P < 0.0001$). A seguito della CDA, si sono ottenute due variabili latenti (CAN 1 e CAN 2) che spiegano il 60% e il 38% della varianza.

Il secondo contributo sperimentale (Blood parameters modification at different ruminal acidosis conditions; capitolo 4) ha dimostrato che per la valutazione dello stato di acidosi ruminale può essere sufficiente un solo rilievo di sangue al giorno, stante la mancanza di differenze statisticamente significative per tutte le variabili ematologiche determinate nel confronto tra i due tempi di campionamento (08:00 vs. 12:00).

Il terzo contributo (Effect of feeding fine maize particles on the reticular pH, milk yield and composition of dairy cows; capitolo 5) ha previsto l'utilizzo di una differente tipologia di boli che hanno la caratteristica di rimanere sul fondo del reticolo. Questi boli hanno registrato in continuo il pH e la temperatura reticolare ed è stato possibile valutare se la somministrazione di mais finemente macinato (Fg) potesse causare un maggiore rischio di SARA in vacche da latte ad alta produzione. Dodici vacche Frisone sono state assegnate a uno dei due gruppi sperimentali: Ct (mais macinato a 1.0 mm) o Fg (mais macinato a 0.5 mm) utilizzando un disegno sperimentale a cross over (2 diete × 2 periodi) basato su una fase di adattamento di 14 giorni e una di rilievo dati di 7 (periodo di 21 giorni). Il pH e la temperatura reticolare sono stati misurati in continuo in 8 vacche utilizzando i boli durante tutta la prova sperimentale. I dati raccolti sono stati sottoposti ad analisi statistica secondo un modello MIXED. Riducendo le dimensioni delle particelle di mais si è osservato un appena significativo aumento dell'ingestione (19.0 vs.

20.3 kg/d per Ct e Fg, rispettivamente; *P* = 0.067), un significativo incremento del livello di proteina grezza (3.18 vs. 3.31%; *P* = 0.021) e caseina nel latte (2.48 vs. 2.57%; *P* = 0.035); per contro, si è osservata una significativa riduzione dell'efficienza alimentare (1.63 vs. 1.52; *P* = 0.008) benché sia aumentata significativamente la digeribilità dell'amido (0.94 vs. 0.98; *P* = 0.078). Le vacche alimentate con Fg hanno significativamente speso maggior tempo sotto la soglia di pH 5.5 (15 vs. 61 min/giorno; *P* = 0.047), hanno avuto una più alta variazione giornaliera media del pH reticolare caratterizzata da un più basso pH nadir (5.95 vs. 5.72; *P* < 0.001) e un più alto range di pH (0.79 vs. 0.94; *P* = 0.003). I trattamenti non hanno influenzato i parametri della temperatura reticolare e neppure il tempo speso giornalmente sotto le soglie di temperatura. Questo studio ha dimostrato che anche le dimensioni delle particelle di mais devono essere prese in considerazione durante la formulazione di una razione volta a massimizzare produzione e qualità del latte, stante il fatto che una loro riduzione sembra favorire un aumento del tenore proteico del latte a scapito di una seppur modesta diminuzione dell'efficienza alimentare; tuttavia, si deve porre attenzione a non aumentare il rischio di acidosi ruminale.

La seconda parte della tesi ha avuto lo scopo di individuare alcune strategie per prevenire SARA nelle vacche da latte tramite l'utilizzo di alcuni supplementi alimentari e di cercare di identificare i meccanismi d'azione attraverso i quali questi agiscono sulla fermentazione ruminale (capitoli 6, 7 e 8). Il quarto contributo sperimentale (Effects of carbohydrase inhibiting compounds on *in vitro* rumen fermentation; capitolo 6) è stato uno studio *in vitro* sulla fermentazione ruminale che ha previsto l'utilizzo di derivati vegetali (mirtillo, faseolamina, gelso bianco e lino comune) a due dosi (15 o 150 mg) aggiunti a 0.5 g di farina di mais (mais macinato attraverso un setaccio di 2 mm). Gli

additivi hanno dimostrato sia di poter modificare il pattern fermentativo sia di poter influenzare la composizione batterica *in vitro* stante le differenze nella concentrazione di azoto ammoniacale e nelle proporzioni di acidi grassi volatili. In particolare, rispetto a un controllo positivo (acarbosio), mirtillo e gelso hanno determinato una maggiore caduta di pH probabilmente a causa della rapida fermentazione del loro contenuto in zuccheri. Il mirtillo inoltre ha aumentato il propionato e comportato un apparente calo della concentrazione di azoto ammoniacale; il mirtillo sembra avere la capacità di limitare la degradazione dell'amido mascherato, sebbene questo effetto sia stato limitato dalla fermentazione degli zuccheri presenti nel supplemento**.**

Negli ultimi due contributi (Use of dicarboxylic acids and polyphenols to attenuate reticular pH drop and acute phase response in dairy heifers fed a high grain diet; capitolo 7. Effect of dicarboxylic acids and polyphenols on rumen microbial population in dairy heifers fed a high grain diet though metagenomic analysis; capitolo 8) acidi dicarbossilici (fumarato-malato) ed estratti di piante (miscela di olii essenziali e di polifenoli) sono stati utilizzati per una prova *in vivo* allo scopo di determinare i loro effetti sulla caduta del pH ruminale, sui metaboliti e gli indicatori infiammatori del sangue e del liquido ruminale in manze alimentate con diete ad alto contenuto di mais (capitolo 7) e di valutare i loro effetti sulla popolazione batterica ruminale (capitolo 8). Secondo un protocollo sperimentale a quadrato latino 3 × 3 (3 diete × 3 periodi) sei manze Frisone non gravide sono state alimentate con una dieta a basso contenuto d'amido (LS) per 14 giorni (NDF 39.8%, amido 24.0% SS), seguita da una dieta ad alto contenuto d'amido (HS) per 8 giorni (NDF 33.6%, amido 30.0% SS). Durante la somministrazione della dieta HS gli animali sono stati assegnati a uno dei tre trattamenti: nessun supplemento/controllo (CT), una dose giornaliera di 60 g al giorno di una miscela di fumarato-malato (FM), o 100 g al

giorno di una miscela di olii essenziali e polifenoli (PM). Il pH ruminale è stato misurato in continuo mediante boli fissi all'interno del reticolo e confrontato con quello rilevato tramite un pH-metro portatile sul liquido ruminale raccolto mediante ruminocentesi il 21° giorno di ogni periodo sperimentale alle 14:00. Un'aliquota di liquido ruminale è stata raccolta per studiare gli effetti dei supplementi sulla produzione di acidi grassi volatili (AGV) e lipopolisaccaridi (LPS) e sulla popolazione microbica ruminale utilizzando la real-time PCR (qPCR) e il sequenziamento Illumina (capitolo 8). Lo stesso giorno sono stati inoltre raccolti campioni di sangue (08:00) e feci (08:00, 14:00 e 21:00). Il coefficiente di correlazione tra i valori di pH acquisiti tramite i boli reticolari o la ruminocentesi è stato pari a 0.83 ($P < 0.001$). Il pH nadir è risultato più basso nella dieta controllo (5.40, 5.69 e 5.62 per CT, FM e PM, rispettivamente; $P = 0.037$), confermando l'efficacia di entrambi i supplementi nel ridurre la caduta del pH ruminale causata da alimenti ricchi di cereali. Inoltre, PM ha dimostrato di essere più efficace rispetto a FM nel ridurre la risposta infiammatoria con una diminuzione delle concentrazioni di neutrofili (2.9, 3.2 e 2.8 10^9/L; $P = 0.084$), proteine della fase acuta come siero amiloide A (37.1, 28.6 e 20.1 μg/mL; $P = 0.036$), LBP (4.1, 3.8 e 2.9 μg/mL; $P = 0.048$) e aptoglobina (675, 695 e 601 μg/mL; $P = 0.084$). Il pH e la concentrazione di lipolisaccaridi nelle feci non sono state influenzate dai trattamenti, mentre i valori di pH nelle feci sono risultati influenzati dal tempo di raccolta (6.38, 6.71 e 6.69 alle ore 08:00, 14:00 e 21:00, rispettivamente; $P = 0.042$) (capitolo 7). I polifenoli hanno evidenziato un maggiore numero di specie osservate ($P < 0.10$), un maggior indice Chao1 ($P < 0.05$), abundance based coverage estimated (ACE) ($P < 0.05$), e Fisher's alpha diversity ($P < 0.10$). Gli acidi organici hanno mostrato valori intermedi tra CT e PM, ad eccezione di Chao1. Con l'unweighted Unifrac distance, l'analisi delle componenti principali (PCoA),

si è ottenuta una significativa separazione tra controllo e polifenoli (P = 0.05) mentre la separazione è risultata modesta tra i supplementi (P = 0.09). Entrambi i supplementi (acidi organici e polifenoli) hanno favorito un significativo aumento di abbondanza relativa della famiglia *Christenenellaceae* e un decremento di *Prevotella brevis* rispetto al controllo. Inoltre, i polifenoli hanno significativamente migliorato l'abbondanza relativa di molti taxa appartenenti ai phyla *Bacteroidetes*, *Firmicutes* e *Tenericutes* probabilmente dovuta a un'attività antimicrobica dei flavonoidi che portano a una competizione tra batteri (capitolo 8).

Concludendo, strumenti diagnostici (diretti e indiretti) come boli e variabili del sangue sono risultati essere promettenti per monitorare variazioni di pH ruminale. Tali strumenti diagnostici possono essere facilmente utilizzati negli allevamenti di vacche da latte ad alta produzione. Per quanto riguarda inoltre la prevenzione di SARA, tra gli additivi alimentari testati, gli acidi dicarbossilici (fumarato-malato) e soprattutto i polifenoli si sono dimostrati efficaci nell'attenuare la caduta del pH ruminale dovuta all'uso di diete ad alto contenuto di cereali, agendo sulla popolazione microbica del rumine. Tali risultati suggeriscono il loro utilizzo negli allevamenti per aiutare a garantire la salute e il benessere delle bovine da latte, favorendo di conseguenza produttività e redditività per gli allevatori.

Summary

Ruminal acidosis is a digestive disorder in dairy and beef cattle and the current definitions are based on the pH of rumen fluid and may occur as an acute, chronic or subclinical (sub-acute) condition. With the aim to achieve the increase of production of milk, highly productive dairy cows are fed cereal-based and energy-dense diets and the rapid ruminal fermentation of starch and sugars leads to an accumulation of volatile fatty acid (VFA) and/or lactate and reduced rumen buffering which cause a drop of pH. Subacute ruminal acidosis (SARA) is characterized by a spontaneous recovery of repeated bouts of low ruminal pH, transient or no accumulation of ruminal lactate, and subtle clinical signs during low ruminal pH bouts. SARA causes consistent direct and indirect economic losses in dairy farming due to the decrease of quality and quantity of milk, increased veterinary costs, premature and involuntary culling. The clinical signs of SARA in the individual cow are subtle, delayed for weeks or months after acidotic insult, not-pathognomonic, and they can be seen in a number of the other diseases. Moreover, ruminal pH varies considerably by different factors like: time after feeding, time of the day, site of sampling within the rumen, techniques of sampling, and individual characteristics. These reasons and the problems in obtaining representative rumen fluid samples make that SARA is under-diagnosticated. Currently, the measurement of rumen pH is used to diagnose SARA, but some diagnostic techniques are invasive (rumen cannulation), or mild invasive (rumenocentesis), give spot sampling of rumen fluid pH decreasing the sensibility on diagnosis of rumen acidosis (rumenocentesis, oral-stomach tube) or cause saliva contamination that alters the values of ruminal pH (oral-stomach tube). So the aim of thesis was the study of easy, rapid and no invasive and economic

direct and indirect diagnostic methods in order to diagnose SARA and indicate the severity of the ruminal acidosis (chapters 3 and 4). Moreover, the effectiveness of different methods of prevention of ruminal acidosis was tested (chapters 5, 6, 7 and 8).

The first experimental contribution of the thesis (Effect of induced ruminal acidosis on blood variables in heifers; chapter 3) was carried out according to a 3 × 3 Latin square arrangement (3 dietary treatments × 3 periods). Six crossbred Valdostana x Belgian Blue non–pregnant heifers were randomly assigned to one of three dietary treatments with different starch levels (% DM): control (CT) as low starch (17.3%), medium starch (MS) for SARA (33.4%), and high starch (HS) to induce acute ruminal acidosis (42.8%). The animals were fed three times a day at 0800, 1200 and 1800 h. Ruminal pH was continuously measured every 10 minutes using wireless sensors featured to fluctuate into the rumen and by rumenocentesis performed at 4 hours after total mixed ration distribution on the 4th day of each experimental period. The regression coefficient between the two methods (wireless sensors vs. rumenocentesis) was 0.56 (P = 0.040). Feeding the CT, MS and HS led to differences in the time spent below the 5.8, 5.5 and 5.0 pH thresholds and in several variables, including dry matter intake (7.7 vs. 6.9 vs. 5.1 kg/d for CT, MS, and HS, respectively; P = 0.002), ruminal nadir pH (5.69 vs. 5.47 vs. 5.44; P = 0.042), mean ruminal pH (6.50 vs. 6.34 vs. 6.31; P = 0.012), haemoglobin level (11.1 vs. 10.9 vs. 11.4 g/dL; P = 0.010), platelet count (506 vs. 481 vs. 601 K/μL; P = 0.008), HCO_3^- (31.8 vs. 31.3 vs. 30.6 mmol/L; P = 0.071) and lipopolysaccharide-binding proteins (5.9 vs. 9.5 vs. 10.5 μg/mL; P < 0.001). Applying a canonical discriminant analysis (CDA), five plasma variables (hemoglobin, mean platelet volume, β-hydroxybutyrate, glucose, and reduced hemoglobin) were able to discriminate among physiological ruminal status: SARA or acute ruminal acidosis. The CDA was

characterized by two significant (Wilks' $\lambda = 0.282$, F approx = 3.76, df1 =15, df2 = 97, $P < 0.0001$) axes, which accounted for 60% and 38% of the existing variation.

The second experimental contribution (Blood parameters modification at different ruminal acidosis conditions; chapter 4) showed that the metabolic condition related to the ruminal acidosis can be evaluated with one daily blood sample because no differences were observed on blood sampling time (8:00 and 12:00 h).

The third experimental contribution (Effect of feeding fine maize particles on the reticular pH, milk yield and composition of dairy cows; chapter 5) evaluated whether feeding finely ground maize (Fg) could cause an increased risk of SARA in high-producing dairy cows. The pH measures were performed through the use of wireless sensors featured to remain in the reticulum, continuously recording reticular pH and temperature. Twelve Holstein-Friesian cows were assigned to one of two experimental groups and were exposed to one of two dietary treatments (maize meal ground to 1.0 mm, control group (Ct) and maize meal ground to 0.5 mm, Fg group) in a 2 × 2 cross-over design (2 diets × 2 periods) over a period of 21 days (14 days of an adjustment phase followed by 7 days of data collection). The pH and temperature of the reticulum were continuously measured in eight cows using indwelling sensors throughout the trial. Data were submitted to statistical analyses according to a mixed model procedure. Reducing the maize particle size, greater dry matter intake (19.0 vs. 20.3 kg/d, for Ct and Fg, respectively; $P = 0.067$), an increase in milk protein (3.18 vs. 3.31%; $P = 0.021$), milk casein (2.48 vs. 2.57%; $P = 0.035$), a reduction in feeding efficiency (1.63 vs. 1.52; $P = 0.008$), and an increase in starch digestibility (0.94 vs. 0.98; $P = 0.078$) were observed. The cows fed Fg diet spent a significantly higher time below the 5.5 pH threshold (15 vs. 61 min/day; $P = 0.047$), had an average daily variation in reticular pH characterized by a

lower nadir pH (5.95 vs. 5.72; $P < 0.001$) and a higher pH range (0.79 vs. 0.94; $P = 0.003$). Neither reticular temperature parameters nor the daily time spent below the temperature thresholds were affected by dietary treatments. This study demonstrated that the grain particle size should be carefully considered during the ration formulation to maximize the production and quality without excessively increasing the risk of ruminal acidosis, since reducing the maize particle size resulted in a drop in the reticular pH, an increase in milk protein content and a slight reduction in feeding efficiency.

The second part of the thesis was to identify prevention strategies against SARA in dairy cows using some feed supplements which could help to prevent SARA and identify the mechanisms of action through which they act on ruminal fermentation (chapters 6, 7, and 8). In the fourth experimental contribution (Effects of carbohydrase inhibiting compounds on *in vitro* rumen fermentation; chapter 6) an *in vitro* rumen fermentation study used plant-derived supplements (bilberry, phaseolamin, white mulberry, and common flax) at two different doses (15 mg and 150 mg) that were added to 0.5 g of maize meal (maize grain ground through a 2-mm screen). The supplements showed to modify fermentation pattern and suggested having effect on the microbial composition *in vitro* given the differences in ammonia N concentration and VFA proportion. In particular, compared to positive control (acarbose), bilberry and mulberry caused the highest drop in pH due to the rapid fermentation of their sugar content. In addition, bilberry resulted in an increase in propionate and in an apparently lower ammonia N concentration and showed an activity against starch degradation, although this effect was concealed by the fermentation of sugars present in the supplement.

In the last two experimental contributions (Use of dicarboxylic acids and polyphenols to attenuate reticular pH drop and acute phase response in dairy heifers fed a high grain diet;

chapter 7 and Effect of dicarboxylic acids and polyphenols on rumen microbial population in dairy heifers fed a high grain diet though metagenomic analysis; chapter 8) the dicarboxylic acids (fumarate-malate) and natural plant extracts (polyphenol-essential oil mixture) were used in an *in vivo* study with the aim to determine their effects in attenuating the drop of rumen pH and the changes in metabolites and inflammatory markers in blood and rumen fluid in dairy heifers fed high-grain diet (chapter 7) and evaluate the changes on the rumen bacterial population (chapter 8). In the fifth and sixth experimental contributions according to a 3 × 3 Latin square experiment (3 dietary treatments × 3 periods) six Holstein-Friesian non-pregnant heifers were fed a low starch (LS) diet for 14 d (NDF 39.8%, starch 24.0% DM), followed by a high starch (HS) diet for 8 d (NDF 33.6%, starch 30.0% DM). During HS feeding, all animals were randomly assigned to one of the following three dietary treatments: no supplement/control (CT), a daily dose of 60 g/d of a fumarate-malate mixture (FM), or 100 g/d of polyphenol-essential oil mixture (PM). Reticular pH was continuously measured using the reticular wireless boluses and on rumen fluid collected by rumenocentesis (1400 h) on d 21 of each period together with blood (0800 h) and fecal samples (0800, 1400, and 2100 h). An aliquot of rumen fluid was collected to study the effects of the supplements (FM and PM) on the rumen bacterial populations using quantitative real-time PCR (qPCR) and Illumina sequencing (chapter 8). The correlation coefficient (r) between the pH values obtained using the reticular boluses and rumenocentesis was 0.83 ($P < 0.001$). Nadir pH was lowest during CT treatment (5.40, 5.69, and 5.62 for CT, FM, and PM, respectively; $P = 0.037$), confirming the effectiveness of both supplements in reducing the pH drop caused by high maize feeding. Moreover, the PM treatment demonstrated to be more effective than FM treatment in reducing the inflammatory response compared to CT treatment with

a decrease of the concentrations of neutrophils (2.9, 3.2, and 2.8 10^9/L; $P = 0.084$), acute phase proteins as serum amyloid A (37.1, 28.6, and 20.1 μg/mL; $P = 0.036$), lipopolysaccharide-binding proteins (4.1, 3.8, and 2.9 μg/mL; $P = 0.048$), and haptoglobin (675, 695, and 601 μg/mL; $P = 0.084$). The pH and lipopolysaccharide concentration of feces were not affected by dietary treatment, whereas fecal pH values were affected by time ($P = 0.042$) and were 6.38, 6.71, and 6.69 at 0800, 1400, and 2100 h, respectively. The polyphenols treatment led to the highest number of observed species ($P < 0.10$), Chao1 index ($P < 0.05$), abundance based coverage estimator (ACE) ($P < 0.05$), and Fisher's alpha diversity ($P < 0.10$); whereas the organic acid treatment had intermediate values between control and polyphenols with the exception of Chao1. Principal coordinate analysis (PCoA) with unweighted Unifrac distance showed a low separation among dietary treatments ($P = 0.09$), and a significant separation between the control and polyphenol treatments ($P = 0.05$). Both supplements showed a significant increase of the family *Christenenellaceae* and a decline of *Prevotella brevis* compared to control. Additionally, polyphenols significantly enhanced the abundance of many taxa belonging to *Bacteroidetes*, *Firmicutes* and *Tenericutes* phyla due to antimicrobial activity of flavonoids that widened competition among bacteria.

In conclusion, direct and indirect diagnostic methods such as blood variables and indwelling boluses resulted a promising diagnostic approach to monitor the ruminal pH changes that may be easily used in intensive dairy farms. Regarding the prevention, some feed supplements such as fumarate-malate and polyphenols attenuate the effects of diets with high concentration of cereals acting on ruminal microbial population that may lead to positive effects on rumen fermentations with effects on animal health and welfare, productivity and profitability in intensive dairy farms.

CHAPTER 1: General introduction

1.1 Definition

Ruminal acidosis is a digestive disorder in the dairy and feedlot sectors and the current definitions are based on the pH of rumen fluid and may occur as an acute, chronic or subclinical (sub-acute) condition (Kleen et al., 2003; Stone, 2004; Duffield et al., 2004; Plaizier et al., 2009a; Marchesini et al., 2011). Acute and subacute ruminal acidosis have similar aetiology but clinical diseases are different (Krause and Oetzel, 2006). Uncompensated decline in ruminal pH, accumulation of ruminal lactate and evident clinical signs in affected cows by acute ruminal acidosis are detected; while in subacute ruminal acidosis (SARA) spontaneous recovery of repeated bouts of low ruminal pH, transient or no accumulation of ruminal lactate, and subtle clinical sings during low ruminal pH are observed (Beauchemin and Penner, 2009; Oetzel, 2010). Recently, Calsamiglia et al. (2012) proposed that SARA should be redefined as a high-concentrate syndrome because both the changes in pH and the effect of type of diet are included. SARA causes consistent economic losses in dairy farming that affects their rumen fermentation, health and production (Kleen et al., 2003; Plaizier et al., 2009a), primarily due to the decreased efficiency of milk production, reduced milk fat yield, increased veterinary costs, premature and involuntary culling and increased losses as a result of death (Nordlund et al., 1995; Kleen et al., 2003; Krause and Oetzel, 2006; Enemark, 2008). SARA is estimated to cost the U.S. dairy industry between US$ 500 million to US$ 1 billion a year (Donovan, 1997). Stone et al. (1999) estimated lost income of US$

400 to US$ 475 per cow per year and US$ 1.12 per cow per day from lost production in a 500-cow herd diagnosed with SARA. In this study Stone et al. (1999) demonstrated that an increased ruminal pH, caused a milk production increase by 2.7 kg/d, and milk fat and protein increase by 0.3 and 0.1 percentage points, respectively, that resulted in an increased monthly income of US$ 20,000 for the dairy. An Italian study (Formigoni, 1998) showed that health problems associated with excessive fluctuations of ruminal pH can cost as much as about 260 euros for cow/year.

1.2 Prevalence

Due to the different diagnostic techniques used to collect the samples and different established thresholds to define SARA, data on the prevalence are not easy to compare (Duffield et al., 2004; Plaizier et al., 2009a). Furthermore, due to lack of pathognomonic signs and problems to collect representative rumen fluid samples, SARA is under-diagnosed by veterinarians (Duffield et al., 2004; Gianesella et al., 2010b). However, several studies have been carried out on the prevalence of rumen acidosis in cows in Italy (Morgante et al., 2007), Germany (Kleen et al., 2013), Ireland (O'Grady et al., 2008), Iran (Tajik et al., 2009), USA (Garrett et al., 1997; Oetzel et al., 1999), and Australia (Annison, 2007) (Table 1). From these studies a prevalence of up to about 50% of animals tested has been recorded (Kleen et al., 2013). SARA is present in dairy herds independent from management type, production or stage of lactation of the individual animal (Kleen et al., 2013). However, if Kleen et al. (2009) observed that stage of lactation does not affect in any significant way on SARA prevalence, Garrett et al. (1997) observed differences in prevalence between early lactation cows and mid-lactation cows.

Table 1. Prevalence (%) of SARA at farm and individual levels in different countries.

Prevalence	Country	References
Farms		
42%	Germany	Kleen et al., 2013
27.6%	Iran	Tajik et al., 2009
3 - 10%	Australia	Annison et al., 2007
Individuals		
0 - 58%	Germany	Kleen et al., 2013
11% in grazing dairy cows	Ireland	O'Grady et al., 2008
33%	Italy	Morgante et al., 2007
20.1% of early and peak lactation cows	USA	Oetzel et al., 1999
19% of early lactation cows 26% of mid-lactation cows	USA	Garrett et al., 1997

1.3 Pathogenesis

During recent years the production of milk has been increasing and with the aim to achieve this increase, highly productive dairy cows are fed cereal-based and energy-dense diets and the rapid ruminal fermentation of starch and sugars leads to an accumulation of VFA and/or lactate and reduced rumen buffering, which cause a drop of pH (Kleen et al., 2003; Stone, 2004; Krause and Oetzel, 2006; Plaizier et al., 2009a; Khafipour et al., 2009b). When switching from a dry-period (a low-energy high-forage diet) to an early lactation diet (a high-energy early lactation diet), the sudden increase of short-chain fatty acids (SCFA) levels exceeds the rate at which they can be absorbed across the ruminal epithelium as the papillae are too short and the resorbing surface too small (Nordlundet al., 1995; Esposito et al., 2014). For this reason, at the herd level acidosis commonly occurs in cows in early lactation (Nocek, 1997; Enemark, 2008). Moreover, other groups at risk are cows in mid-lactation which, due to their high feed

intake, are particularly sensitive to sudden changes of feed or faults in feed composition and delivery (Nordlund et al., 1995; Enemark, 2008) and primiparous cows (Krause and Oetzel, 2006). Although, Maekawa et al. (2002) showed a greater risk of SARA in cows with high feed intake.

1.4 Clinical signs

The clinical signs of SARA in the individual cow are subtle, delayed for weeks or months after acidotic insult and not-pathognomonic and can be seen in a number of the other diseases (Krause and Oetzel, 2006; O'Grady et al., 2008). Clinical signs of SARA may be most identifiable as a herd profile (Nordlund and Garret, 1994) and in the differential diagnosis of herd profile, acidosis should be considered when includes decrease in dry matter intake (DMI), low body condition score (Kleen et al., 2013), laminitis (Nocek et al., 1997; Enemark et al., 2002), caudal vena cava syndrome (Nordlund et al., 1995), abomasal displacement/ulceration (Olson, 1991), hemoptysis and epistaxis (Kleen et al., 2003), abscesses (Kleen et al., 2003), intermittent diarrhea, mastitis and endometritis (Enemark et al., 2002), low fertility (Britt, 1995), depression of milk-fat percentage (Enjalbert et al., 2008; Guo et al., 2013) and high herd cull rates for poorly defined health problems (Nordlund and Garret, 1994). Although decrease of DMI has been used as a clinical sign to diagnose SARA, however SARA induced by alfalfa pellets did not show to reduce DMI (Khafipour et al., 2009a) as SARA induced by grain (Gozho et al., 2005, 2006) even though both led to similar rumen pH, rumen volatile fatty acids (VFA) and osmolarity (Khafipour et al., 2009a).

1.5 Diagnosis

Currently, the measurement of the ruminal pH is used to the diagnose SARA (Nordlund et al., 1995; Enemark et al., 2002; Duffield et al., 2004). Rumenocentesis (Nordlund and Garret, 1994), oral-stomach tube (Duffield et al., 2004) and rumen cannulation (Dado and Allen, 1993; Penner et al., 2006) are inexpensive tools used to obtain spot sampling of rumen fluid. Indwelling boluses are used to continuously monitor the rumen-reticular pH (Dado and Allen, 1993; Penner et al., 2006). Rumenocentesis is considered a mildly invasive technique and some authors have reported problems such as abscesses and peritonitis (Nordlund and Garret, 1994; Aceto et al., 2000) and it is considered by veterinaries not easy to use in routine clinical investigations (Gianesella et al., 2010a). With oral-stomach tube pH varies according to intra-ruminal localization of the stomach tube, saliva contamination and time of sampling in relation to feeding (Enemark, 2008). The rumen cannulation uses fistulated cows but is impractical to cannulate cows in a dairy herd for monitoring rumen fermentation (European Communities, 1998; Li et al., 2013). However, the boluses have some disadvantages: they are expensive, the lifespan is limited (from months to years according to companies) and their removal requires surgery or slaughter. Diagnosis of SARA needs standardization of the timing of rumen fluid collection and the threshold for SARA because sometimes the coefficient of variation among animals is often used as a criterion to determine whether SARA is present (Garrett et al., 1999). Some studies defined a threshold of rumen pH such as between pH 5.2 and 5.6 for at least 3 h/day (Gozho et al., 2005), <5.8 for more than about 5 h/day (Zebeli et al., 2008 or 6 h/day (Zebeli et al., 2012) and between pH 5.2 and 5.6 (Cooper et al., 1999) or <5.8 without a specific duration (Beauchemin et al., 2003). Moreover, ruminal pH varies considerably by:

- Time after feeding (Krause and Oetzel, 2005): with a total mixed ration offered to herds twice a day the lowest pH values occur 5 to 8 h post feeding and when rations are fed as separate components occur 2-5 hours following the primary concentrate meal (Nordlund and Garrett, 1994; Duffield et al., 2004).
- Time of the day due to the processes of eating, rumination, ruminal digestion, and VFA absorption (Dohme et al., 2008; Plaizier et al., 2009a).
- Individual sensitivity of cows (Krause and Oetzel, 2005).
- Site of sampling within the rumen: the rumen pH is highest in the cranial dorsal sac due to buffering of saliva, followed by the cranial ventral, caudal ventral, and the caudal dorsal sac. Rumen pH is the lowest in the ventral sac and in the centre of rumen solid mat (Duffield et al., 2004, Shen et al., 2012).
- Sampling technique: ruminal fluid pH collected by a stomach tube was on average 0.35 pH units higher than rumen fluid collected by rumenocentesis (Duffield et al., 2004). Garrett et al. (1999) showed that ruminal fluid pH was 0.28 pH units lower for fluid collected by rumenocentesis than for fluid collected through a ruminal cannula. Thresholds proposed for indicating SARA are 5.5, 5.8 and 5.9 when rumen fluid samples are collected by rumenocentesis, through a rumen cannula from the ventral sac and using an oral probe, respectively (Plaizier et al., 2009a).

Indirect variables have been used to predict the ruminal pH based on symptoms and laboratory parameters (Enemark et al., 2004; Gozho et al., 2005; Enemark, 2008; Dong et al., 2011).

Some indirect measurements were evaluated as possible indicators to predict SARA:

- Blood: decreased base excess in steers (Brown et al., 2000), pH, pO_2 (Brown et al., 2000; Morgante et al., 2009; Gianesella et al., 2010a), oxygen content plasma (Morgante et al., 2009; Gianesella et al., 2010a), increase in concentrations of pCO_2 (Morgante et al., 2009; Gianesella et al., 2010a), HCO_3^- (Morgante et al., 2009; Gianesella et al., 2010a), oxyhemoglobin (O_2Hb), base excess of extracellular fluid (Morgante et al., 2009), β-hydoxybutyric acid, cholesterol and minerals (Ca, Fe, and Zn) (Ametay et al., 2009; Zebeli et al., 2010; Dong et al., 2011), glucose (Ametaj et al., 2009; Khafipour et al., 2009b), nonesterified fatty acid (NEFA) (Ametaj et al., 2009), neutrophils and acute phase proteins such as serum amyloid A, lipopolysaccharide binding protein (LBP), C-reactive protein and occasionally haptoglobin (Gozho et al., 2007; Khafipour et al., 2009b; Dong et al., 2011; Li et al., 2012b).
- Rumen: the increase in the concentration of lipopolysaccharide (LPS) (Plaizier et al., 2012), temperature (Al-Zahal et al., 2008), osmolality (Owens et al., 1998), propionate (Li et al., 2012a), valerate (Enemark et al., 2004; Morgante et al., 2007), changes in the morphology of the rumen wall (Steele et al., 2011; Mirmazhari-Anwaret et al., 2013), microbial composition with an increment of starch-fermenting bacteria (Stewart et al., 1997; Nagaraja and Titgemeyer, 2007; Khafipour et al., 2009c) and Gram-negative bacteria (Plaizier et al., 2012).
- Urine: an increase in renal excretion of H+ and a decrease in pH (Owens et al. 1998; Mellau et al., 2004; Gianesella et al., 2010a) and net acid-base excrection (NABE) in herd diagnostic (Enemark, 2008). Although in Li et al. (2012a) and Enemark et al. (2004) urine pH and NABE were increased by alfa-alfa pellet and not by grain diet.

- Faeces: they are bright, yellowish, have a sweet–sour smell (Kleen et al., 2003), foamy with gas bubbles, and contain more than normal amounts of undigested fibre or grain (Hall, 2002; Enemark, 2008) and the concentration of LPS is increased (Plaizier et al., 2009b; Li et al., 2012a,b).
- Milk: reduction in milk yield, milk fat content and protein production, 3.5% fat-corrected milk yield, milk energy efficiency (Stone et al., 1999, 2004; Corato et al., 2005; Marchesini et al., 2009; Dong et al., 2011), milk–urea–nitrogen <3.0 mmol/L (Enemark, 2008), Soxleth-Henkel-figure >8 (Enemark, 2008); fat–protein-ratio <1 (Enemark, 2008); greater concentrations of C11:0, C13:0, C15:0, C14:1, C16:1, C17:1, C18:2n6c, C20:3n6, total polyunsaturated fatty acids (FA) and total odd-chain FA and lower concentrations of C18:0 and total saturated FA (Guo et al., 2013).

1.6 Microbial communities

The induction of ruminal acidosis affects microbial communities in the rumen and in the large intestine (Li et al., 2012b). Low pH changes the rumen microbes, so it is expected that rumen acidosis reduces cellulolytic and gram-negative bacteria and increases populations of gram-positive cocci and rods (Nagaraja et al., 1978; Wells and Russell, 1996; Goad et al., 1998.). In particular, grain-induced ruminal acidosis reduces richness and diversity of bacterial species in the rumen, large intestine and feces (Fernando et al., 2010; Li et al., 2011; Khafipour et al., 2011), increases the populations of starch-fermenting and lactic acid-utilizing bacteria (Nagaraja and Titgemeyer, 2007) and reduces gram-negative such as *Bacteroidetes* bacteria including *Prevotella albensis*, *Prevotella brevis*, and *Prevotella ruminicola* (Khafipour et al., 2009c). An excessive depression in rumen pH causes an increase the lysis of Gram-negative bacteria resulting

in the release of free LPS endotoxins (Plaizier et al., 2009a; Plaizier et al., 2012; Mao et al., 2013). When translocation of free LPS from the rumen and/or gut into portal circulation occurs, an immune response in the host is induced (Dong et al., 2011).

1.7 Prevention

SARA is not often treated because its diagnosis is difficult (Li et al., 2012a), and for this reason the development of prevention strategies is highly desirable (Owens et al., 1998; Enemark, 2008). The major risks of SARA in dairy herd are ruminal buffering caused by inadequate dietary fibre and/or inadequate physical fibre, excessive intake of rapidly fermentable carbohydrates, and inadequate ruminal adaptation to a highly fermentable diet (Krause and Oetzel, 2006). So the prevention of SARA can be obtained providing adequate ruminal buffering such as proper amounts of cations relative to anions and the addition of dietary buffers (e.g. sodium bicarbonate, sodium sesquicarbonate, magnesium oxide, sodium bentonite, calcium carbonate, potassium carbonate) (Enemark, 2008). Adequate physical fibre, encouraging saliva production during chewing and increasing rumination after feeding (long-fiber particles that are <~5 cm in length), wetter rations to minimize sorting in the diets (Heinrichs and Kononoff, 2002; Stone et al., 2004), control the access to concentrates station (EFSA, 2008) and adequate bunk space (0.61 m/cow) to avoid the competition should be guaranteed all the time (Shaver, 2002). Other aspects to prevent SARA are the supply of a controlled amount of rapidly fermentable carbohydrates, processing grains properly, including high fibre concentration as needed and garantering feed to cows all day long to allow them to consume regularly small and frequent meals (Krause and Oetzel, 2006). A gradual adaption to highly fermentable diets permits a microbial adaptation and lengthening of the ruminal papillae (about 4 weeks)

(Nocek et al., 1997). However, different studies (Anderson et al., 1994; Garret et al., 1997) showed no effects of concentrate feeding in the dry-period on early lactation ruminal pH. Although good management practices and nutritional strategies are used, not always they are enough to avoid the onset of SARA in high yielding dairy cows. In alternative to antibiotics, that are banned by the European Community (European Communities, 2003), other preventative measures have been suggested to manipulate rumen microbial community and subsequently ruminal fermentation: physical manipulation of fiber and grain particle size (Enemark, 2008; Marchesini et al., 2011), the use of yeasts, probiotic bacteria such as propionobacteria, lactobacilli and enterococci (Ghorbani et al., 2002; Bach et al., 2007; Long et al., 2014), the addition of dicarboxylic acids (Nisbet et al., 2009), flavonoids (Balcells et al., 2012) or essential oils (Calsamiglia et al., 2007).

CHAPTER 2: General aim

The objective of this thesis was to identify direct and indirect methods to diagnose SARA and indicate the severity of the ruminal acidosis evaluating the variation of these parameters at different sampling times in heifers. Moreover, another aim was to study some plant extracts and feed additives which could prevent SARA and identify the mechanisms of action through which they act on ruminal fermentation. The main aims were:

1. Model the data obtained from the complete blood cell count, biochemical plasma profile, venous blood gas analysis, analysis of blood lipopolysaccharide (LPS) and LPS-binding proteins (LBP) to identify a subset of variables that could reliably indicate the severity of the induced ruminal acidosis in heifers (see chapter 3).
2. Identify the blood variables change in heifers affected by different levels of ruminal acidosis and to evaluate the variation of these parameters at different sampling times (see chapter 4).
3. Determine whether feeding finely ground maize could cause an increased risk of SARA in high-producing dairy cows by continuously recording reticular pH and temperature; the effects of maize particle size on DMI, feed total tract apparent digestibility, milk yield and milk composition were also investigated (see chapter 5).
4. Verify the effectiveness of plant extracts such as bilberry, phaseolamin, white mulberry, and common flax in reducing the ruminal fermentation of maize meal; the degree of fermentation was estimated through the measurement of pH, VFA, changes in ammonia N and the apparent disappearance of dry matter and starch (see chapter 6).

5. Determine in high grain fed dairy heifers the effects of two feed additives, a fumarate-malate mixture and a polyphenol-essential oil mixture, in attenuating the drop of rumen pH and the changes in metabolites and inflammatory markers in blood and rumen fluid (see chapter 7).

6. Determine the effect of the some additives in changing the rumen bacterial population (see chapter 8).

CHAPTER 3

Effect of induced ruminal acidosis on blood variables in heifers

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Effect of induced ruminal acidosis on blood variables in heifers

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Abstract

Background: Ruminal acidosis is responsible for the onset of different pathologies in dairy and feedlot cattle, but there are major difficulties in the diagnosis. This study modelled the data obtained from various blood variables to identify those that could indicate the severity of ruminal acidosis. Six heifers were fed three experimental rations throughout three periods. The diets were characterised by different starch levels: high starch (HS), medium starch (MS) and low starch, as the control diet (CT). Ruminal pH values were continuously measured using wireless sensors and compared with pH measurements obtained by rumenocentesis. Blood samples were analysed for complete blood count, biochemical profile, venous blood gas, blood lipopolysaccharide (LPS) and LPS-binding proteins (LBP).

Results: The regression coefficient comparing the ruminal pH values, obtained using the two methods, was 0.56 (P = 0.040). Feeding the CT, MS and HS led to differences in the time spent below the 5.8, 5.5 and 5.0 pH thresholds and in several variables, including dry matter intake (7.7 vs. 6.9 vs. 5.1 kg/d; P = 0.002), ruminal nadir pH (5.69 vs. 5.47 vs. 5.44; P = 0.042), mean ruminal pH (6.50 vs. 6.34 vs. 6.31; P = 0.012), haemoglobin level (11.1 vs. 10.9 vs. 11.4 g/dL; P = 0.010), platelet count (506 vs. 481 vs. 601; P = 0.008), HCO_3^- (31.8 vs. 31.3 vs. 30.6 mmol/L; P = 0.071) and LBP (5.9 vs. 9.5 vs. 10.5 µg/mL; P < 0.001). A canonical discriminant analysis (CDA) was used to classify the animals into four ruminal pH classes (normal, risk of acidosis, subacute ruminal acidosis and acute ruminal acidosis) using haemoglobin, mean platelet volume, β-hydroxybutyrate, glucose and reduced haemoglobin.

Conclusions: Although additional studies are necessary to confirm the reliability of these discriminant functions, the use of plasma variables in a multifactorial model appeared to be useful for the evaluation of ruminal acidosis severity.

Keywords: Ruminal acidosis, Blood variables, Wireless rumen sensor, Heifers

Background

Ruminal acidosis is an ongoing problem in the dairy and feedlot sectors. It has been shown to cause consistent economic losses in dairy farming, primarily due to the reduction in milk yield and milk fat, premature culling and increased losses as a result of death [1]. In both the beef and dairy industries, many authors [2-5] have reported that ruminal acidosis is responsible for the onset of different pathologies, such as rumenitis, parakeratosis, metabolic acidosis, and laminitis. There are major challenges in improving the understanding of acute ruminal acidosis and subacute ruminal acidosis (SARA), including a wide range of responses observed under identical conditions [6]

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and difficulties in measuring the pH of the rumen, which require procedures such as rumenocentesis, oesophageal intubation or rumen cannulation.

There have been many attempts to use indirect variables to predict the ruminal pH based on symptoms or blood and metabolic indicators [2,7-9]. However, none of the variables alone have predicted the ruminal status of cattle, and only a few of the authors attempted to model metabolic variables to evaluate ruminal acidosis [10].

The aim of this study was to model the data obtained from the complete blood cell count, biochemical plasma profile, venous blood gas analysis, analysis of blood lipopolysaccharide (LPS) and LPS-binding proteins (LBP) to identify a subset of variables that could reliably indicate the severity of the induced ruminal acidosis in heifers.

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Results and discussion

Animal health and body weight

Animal health was not compromised by the experiment as certified by a veterinarian at the end of each period. At the end of the trial, the heifers weighed an average of 382±17.3 kg with an average daily gain of 0.75±0.09 kg/d.

Feed intake

The dry matter intake (DMI) was significantly affected by the treatment, the day, the interactions period x day and treatment x period x day (Table 1). The interactions were significant because the challenge diets were provided only on the challenge day (d1) as specified in the protocol to induce acidosis. The lowest DMI was observed following the high starch (HS) treatment as a result of the ruminal pH drop (Figure 1) on the day after the d1 (d2) and it could be explained as an attempt to avoid the effects of the very low ruminal pH. Moreover, in the second period, the heifers that had experienced a pH below 5.0 after ingesting the MS diet in the first period dramatically reduced their intake with the HS diet (Figure 2). The reluctance to consume diets rich in starch after experiencing ruminal acidosis could be explained as a memory effect due to previously experienced ruminal acidosis despite the two-week recovery period. This result depends not only on the memory effect but also on individual sensitivity to ruminal acidosis. Heifers that consumed MS feed in the second period had less severe acidosis than the heifers that

Table 1 Effects of dietary treatment (T, n = 18) and time period (P, n = 18) on DMI, ruminal pH, blood count, blood gas, haematological profile and acute phase proteins

	Treatment[1]			Period[2]			P-value			SEM
Trait	CT	MS	HS	1	2	3	T	P	T x P	
DMI, kg/d	7.7^{a}	6.9^{a}	5.1^{b}	6.2	6.6	6.9	0.002	0.426	0.571	0.39
Nadir ruminal pH	5.69^{a}	5.47^{ab}	5.44^{b}	5.23^{b}	5.75^{a}	5.62^{a}	0.042	0.003	0.307	0.073
Mean ruminal pH	6.50^{a}	6.34^{b}	6.31^{b}	6.15^{b}	6.48^{a}	6.52^{a}	0.012	0.001	0.116	0.076
Max ruminal pH	7.13	7.09	7.08	6.97^{b}	7.08^{ab}	7.25^{a}	0.423	0.001	0.054	0.090
Blood count and gas										
HGB, g/dL	11.1^{ab}	10.9^{b}	11.4^{a}	11.7^{a}	10.8^{b}	10.9^{b}	0.010	0.001	0.526	0.23
HCT, %	33.8^{ab}	32.8^{b}	34.1^{a}	35.4^{a}	32.3^{b}	33.0^{b}	0.027	<0.001	0.563	0.60
PLT, K/µL	506^{b}	481^{b}	601^{a}	493	564	530	0.008	0.177	0.043	78.4
MPV, fl	4.2	4.1	3.9	4.0	4.2	4.0	0.542	0.840	0.266	0.26
pCO_2, mmHg	52.0	50.3	50.4	50.4	50.9	51.4	0.126	0.502	0.137	0.63
pO_2, mmHg	61.7	72.3	71.1	42.1^{b}	71.4^{ab}	91.6^{a}	0.450	0.003	0.454	7.10
HCO^-_3, mmol/L	31.8$^{\alpha}$	31.3$^{\alpha\beta}$	30.6$^{\beta}$	31.8	31.3	30.7	0.071	0.127	0.081	0.45
O_2Hb, %	87.6	87.9	86.3	77.5^{b}	89.9^{a}	94.5^{a}	0.728	<0.001	0.683	2.01
RHb, %	9.9	9.8	12.4	20.4^{a}	8.6^{b}	3.1^{b}	0.381	<0.001	0.721	1.96
sO_2m, %	89.9	90.3	87.5	79.2^{b}	91.4^{a}	97.0^{a}	0.393	<0.001	0.715	2.12
Haematological profile and acute phase proteins										
Glucose, mmol/L	4.34	4.37	4.32	4.42$^{\alpha}$	4.26$^{\beta}$	4.35$^{\alpha\beta}$	0.686	0.098	0.891	0.104
CHOL, mmol/L	3.52	3.37	3.45	3.27^{b}	3.28^{b}	3.79^{a}	0.446	0.005	0.548	0.130
NEFA, meq/L	0.23	0.20	0.27	0.24	0.21	0.25	0.155	0.624	0.555	0.025
β-HB, mmol/L	0.28	0.31	0.29	0.26$^{\beta}$	0.30$^{\alpha\beta}$	0.31$^{\alpha}$	0.440	0.069	0.406	0.016
AST, U/L	78.2^{ab}	72.1^{b}	82.0^{a}	76.1$^{\alpha\beta}$	74.4$^{\beta}$	81.7$^{\alpha}$	0.007	0.053	0.092	1.70
γGT, U/L	19.2	19.4	18.6	17.7^{b}	19.2^{ab}	20.4^{a}	0.527	0.031	0.622	1.33
LBP, µg/ml	5.9^{b}	9.5^{a}	10.5^{a}	10.5^{a}	7.4^{b}	7.9^{b}	<0.001	0.014	0.221	0.92

[1] CT = control; MS = medium starch; HS = high starch. [2] Experimental periods. $^{a–b}$ Means within a row with different superscripts differ (P < 0.05). $^{\alpha–\beta}$ Means within a row with different superscripts differ (P < 0.10). (HGB = haemoglobin; HCT = haematocrit; PLT = platelet count; MPV = mean platelet volume; pCO_2 = partial pressure of carbon dioxide; pO_2 = partial pressure of oxygen; HCO^-_3 = bicarbonate level; O_2Hb = oxyhaemoglobin; RHb = reduced haemoglobin; sO_2m = measured oxygen saturation; CHOL = cholesterol; β-HB = β-hydroxybutyrate; AST = aspartate aminotransferase; γGT = γ-glutamyl transferase; LBP = lipopolysaccharide-binding protein. The day (D) effect (P) was as follows: DMI (< 0.001), mean pH (< 0.001), max pH (< 0.001), HGB (0.017), total protein (0.015), glucose (< 0.001), CHOL (0.014), NEFA (< 0.001), β-HB (0.012), AST (0.054) and LBP (0.012). The P value was > 0.05 for the other variables tested. The interaction P x D effect (P) was as follows: DMI (0.011), mean pH (0.008), nadir pH (0.015), HGB (0.041), pO_2 (0.049), glucose (0.001) and NEFA (0.026). The P value was > 0.05 for the other variables tested. The interaction T x P x D effect (P) was as follows: DMI (0.008) and mean pH (0.047). The P value was > 0.05 for the other variables tested.

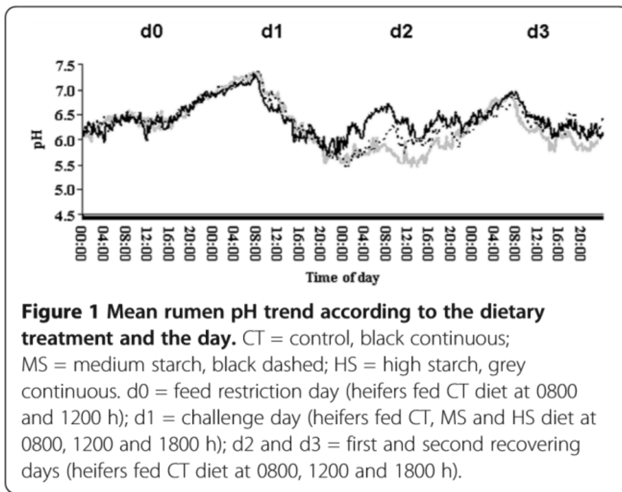

Figure 1 Mean rumen pH trend according to the dietary treatment and the day. CT = control, black continuous; MS = medium starch, black dashed; HS = high starch, grey continuous. d0 = feed restriction day (heifers fed CT diet at 0800 and 1200 h); d1 = challenge day (heifers fed CT, MS and HS diet at 0800, 1200 and 1800 h); d2 and d3 = first and second recovering days (heifers fed CT diet at 0800, 1200 and 1800 h).

had fed on the same diet in the first period (Figure 3) and showed a lower reduction in the intake of HS feed in the third period (Figure 2).

Ruminal pH

The regression coefficient between the ruminal pH values obtained using sensors and rumenocentesis was 0.56 (P = 0.040), indicating a degree of agreement between the two methods.

The control (CT) treatment led to the highest nadir and mean ruminal pH values, whereas the lowest nadir pH level was reported for the HS treatment (Table 1). In the first period, the heifers showed the lowest values of maximum, mean and nadir pH (Table 1). The significant effects of the period and of the interactions treatment x period, period x day and treatment x period x day were due to the variations of DMI during the challenge day (Figure 2) and to individual ability to cope with the dietary factors that predispose animals to acidosis [6].

To evaluate the level of ruminal acidosis, the mean amount of time per day that the pH was below three ruminal pH thresholds (pH < 5.0; 5.0 ≤ pH <5.5 and 5.5 ≤ pH < 5.8) was determined and is reported in Figure 3. The ruminal pH fell below 5.0 during the first period for the HS and MS treatments when the animals experienced acute ruminal acidosis. The heifers feeding on MS during the first period had a pH between 5.0 and 5.5 for the longest period of time, while the pH never dropped below 5.5 in the heifers that were fed the CT treatment in the second and third periods. The pH varied between 5.5 and 5.8, ranging from at least 10 min per day on the CT diet during the third period to 180 min per day on the MS diet during the first period (Figure 3). The large differences in the amount of time the pH was below 5.0 between periods were related to the DMI (Figure 2), individual sensitivity to acidosis and a possible memory effect in the animals that had previously experienced acidosis.

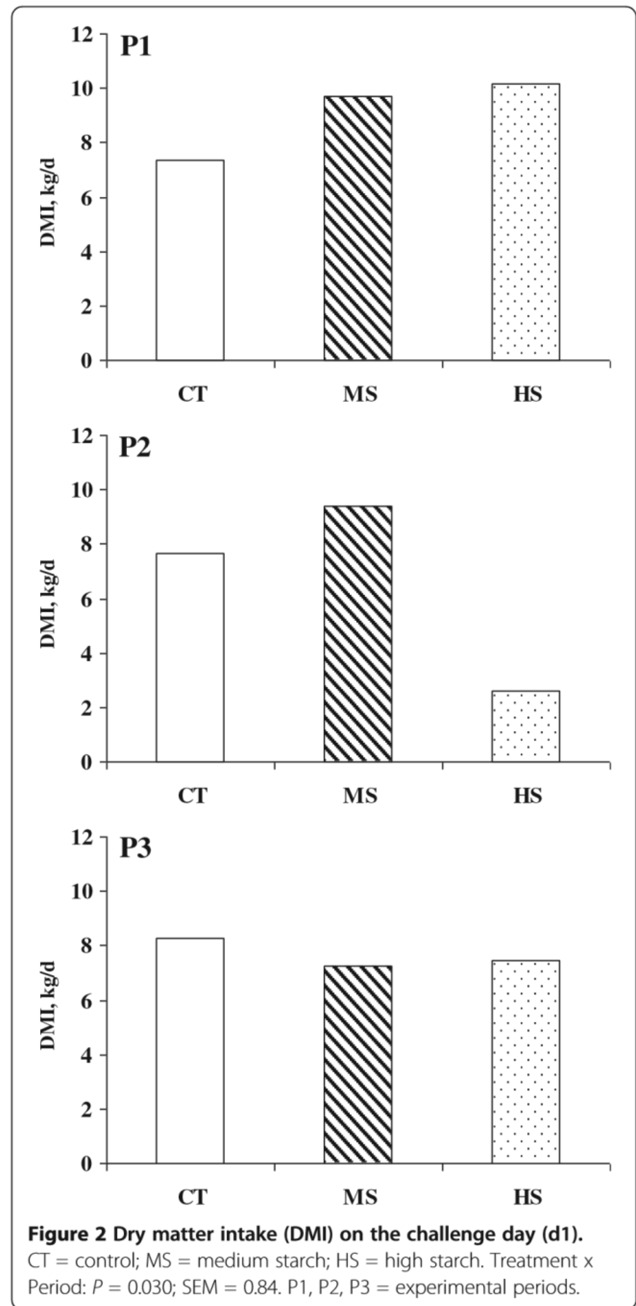

Figure 2 Dry matter intake (DMI) on the challenge day (d1). CT = control; MS = medium starch; HS = high starch. Treatment x Period: P = 0.030; SEM = 0.84. P1, P2, P3 = experimental periods.

Blood analysis

Of the treatments, the heifers fed HS showed the highest concentrations of haemoglobin (HGB), haematocrit (HCT), platelet count (PLT) and aspartate aminotransferase (AST). The LBP was higher in the heifers fed HS and MS, and the bicarbonate (HCO_3^-) level was the lowest in HS. The concentrations of HGB, HCT, and PLT were higher in the heifers that had ruminal acidosis for longer periods of time due to high ruminal osmotic pressure, which pulls fluid from plasma into the rumen and concentrates the blood components [3,11]. PLT could be influenced by the onset of

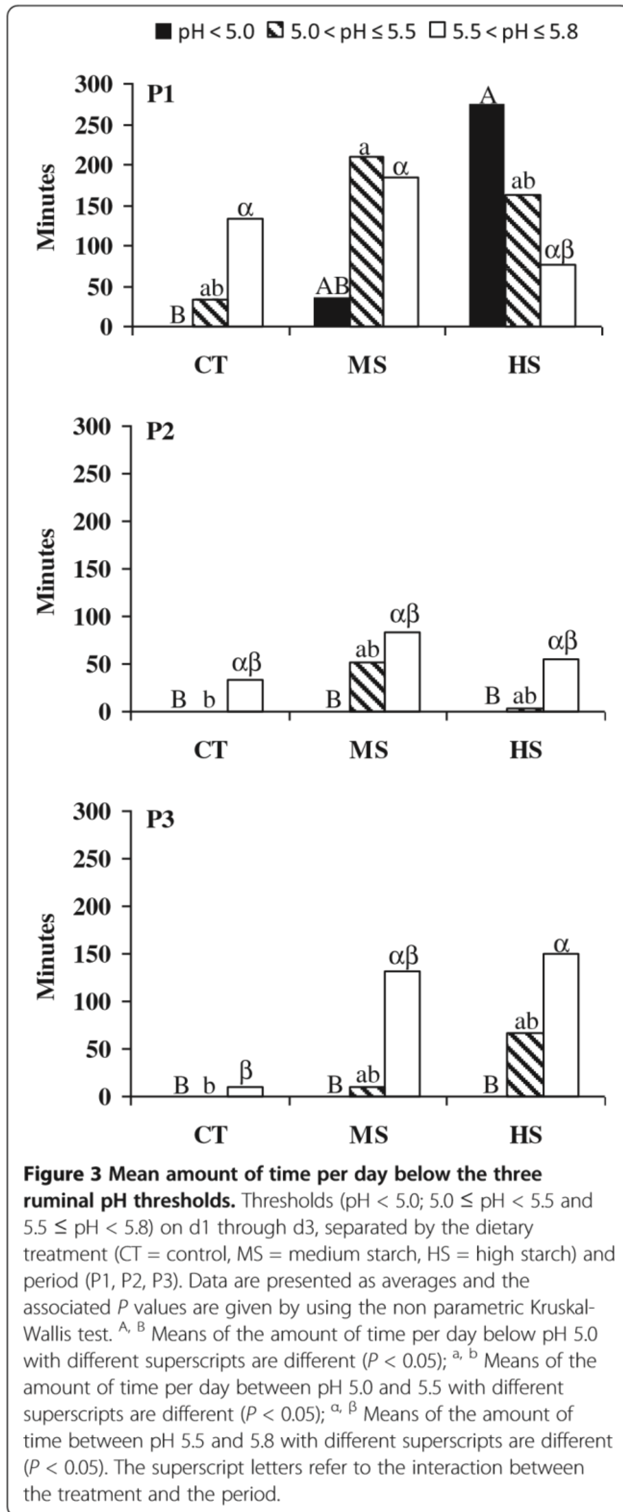

Figure 3 Mean amount of time per day below the three ruminal pH thresholds. Thresholds (pH < 5.0; 5.0 ≤ pH < 5.5 and 5.5 ≤ pH < 5.8) on d1 through d3, separated by the dietary treatment (CT = control, MS = medium starch, HS = high starch) and period (P1, P2, P3). Data are presented as averages and the associated *P* values are given by using the non parametric Kruskal-Wallis test. [A, B] Means of the amount of time per day below pH 5.0 with different superscripts are different ($P < 0.05$); [a, b] Means of the amount of time per day between pH 5.0 and 5.5 with different superscripts are different ($P < 0.05$); [α, β] Means of the amount of time between pH 5.5 and 5.8 with different superscripts are different ($P < 0.05$). The superscript letters refer to the interaction between the treatment and the period.

damage to the rumen mucosa as a result of acidosis, as reported by other authors [5].

The concentration of LPS in the peripheral blood plasma was below the assay detection limit of 0.1 EU/mL for all treatments. This result was likely due to the high clearance rate of the LPS in the Kupfer cells of the liver, which resulted in the absence of LPS in the peripheral blood and caused an inflammation cascade that led to the production of LBP [12].

The higher levels of LBP in the heifers fed MS and HS were due to the high starch intake and reduction in the ruminal pH. Some authors [9] reported that the early hours following grain engorgement are characterised by the rapid growth of Gram-negative bacteria, which undergo cell lysis and release LPS following a reduction in the ruminal pH. The translocation of LPS from the digestive tract to the bloodstream increases the LBP levels as a consequence of the systemic immune response [12]. The clearing of LPS in the liver could explain the slight increase in AST, which is a non-specific liver enzyme [13] that indicates liver alterations. The drop ($P < 0.10$) in HCO_3^- level represents a mechanism to contrast the incoming of metabolic acidosis as a result of ruminal acidosis [14].

In this study, the period significantly affected the blood count, gas composition and the haematological profile (Table 1). The first period, which was characterised by increased amount of time below the established pH thresholds (Figure 3), led to an increase ($P < 0.05$) in HGB, HCT, reduced haemoglobin (RHb) and LBP and showed a slightly higher ($P < 0.10$) level of glucose. During the first period, there was a reduction ($P < 0.05$) in the partial pressure of oxygen (pO_2), oxyhaemoglobin (O_2Hb), measured oxygen saturation (sO_2m), γ-glutamyl transferase (γGT) and a slight ($P < 0.10$) decrease in β- hydroxybutyrate (β-HB), whereas cholesterol (CHOL) was similar to the second period and AST showed an intermediate value.

The variations in RHb, pO_2, O_2Hb and sO_2m reflected the effects of the cellular buffering system, which represents one of the mechanisms to maintain the blood pH within a physiological range as reported in humans [15]. During ruminal lactic acidosis, excess organic acids that accumulate in the rumen are absorbed into the bloodstream at the risk of overwhelming the bicarbonate buffering system [14]. When the blood pH begins to drop in response to decreased HCO_3^- levels, there is a shift in the oxyhaemoglobin dissociation curve and the red blood cells release oxygen to the tissues more readily, which increases the RHb and reduces the O_2Hb, pO_2 and sO_2m [15]. The slightly higher glucose level in the first period was a consequence of the increased DMI of HS and MS on d1 (Figure 2), which were rich in starch and led to a higher absorption of glucose in the small intestine. The low level of β-HB and cholesterol in the first period could be related to an altered energy status in the animals. As reported by other authors [16], the high level of glucose could have lowered the β-HB concentration while the variation in cholesterol levels could be linked to interactions between many factors, including the DMI and the ruminal pH [17].

The highest concentration of AST and γGT in the third period could be due to stress on the liver as a consequence of the considerable variations in dietary patterns during the experiment.

A canonical discriminant analysis (CDA) was applied to the four ruminal acidosis classes (such categorisation is based on the amount of time the pH is below the established pH thresholds). The CDA was characterised by two significant (Wilks' λ = 0.282, F approx = 3.76, df1 = 15, df2 = 97, $P < 0.0001$) axes, which accounted for 60% and 38% of the existing variation. Among all the blood variables, HGB, mean platelet volume (MPV), β-HB, glucose and RHb contributed the most to the discriminant model (Table 2). Contrary to our expectations, LBP, an acute phase protein that was reported to increase during ruminal acidosis [9], was not included in the model even though it was higher in MS and HS compared with CT (Table 1). A possible reason is that LBP showed a different trend between d1 and d3 (7.0, 9.6 and 9.4 μg/ml for d1, d2 and d3, respectively, $P = 0.012$) compared to the pH trend, i.e., nadir pH (5.58, 5.40 and 5.62 for d1, d2 and d3, respectively, $P = 0.084$). The variables selected in the model explain the status of dehydration (HGB), the production of new platelets in the bone marrow, which are possibly due to lesions at the ruminal level (MPV), the energy status (β-HB and glucose) and the activation of the cellular buffering system to maintain the blood pH within a physiological range (RHb). Although single variables cannot predict the presence and severity of ruminal acidosis due to the considerable variation in the ability of an animal to cope with a carbohydrate challenge, evaluating specific combinations of blood variables that can highlight the ongoing processes of adaptation to the ruminal stress in the animal appears to be a promising approach in diagnosing and monitoring ruminal acidosis.

As reported in Figure 4, the scattergram relative to the total canonical structure expressing the correlation of HGB, MPV, β-HB, glucose and RHb with the canonical axes (CAN 1, $P < 0.001$ and CAN 2, $P = 0.009$) showed good separation between the different pH classes, with the exception of the animals classified as normal (N) or at risk of ruminal acidosis (R), which were not distinguished.

Table 2 Summary of the steps for the interactive forward mode (stepwise) for the CDA

	Wilks' λ	P-value
HGB	0.715	0.005
MPV	0.505	<0.001
β-HB	0.420	<0.001
Glucose	0.347	<0.001
RHb	0.280	<0.001

HGB = haemoglobin; MPV = mean platelet volume; β-HB = β-hydroxybutyrate; RHb = reduced haemoglobin.

Squared Mahalanobis distances (D^2-Mahalanobis) obtained using CDA between the ruminal acidosis groups showed that acute ruminal acidosis (A) was different from the SARA (S) (D^2-Mahalanobis = 4.9; $P = 0.002$) , N (D^2-Mahalanobis = 7.1; $P < 0.001$) and R groups (D^2-Mahalanobis = 3.6; $P = 0.010$). SARA showed a significant separation from the R ($P = 0.017$) and N ($P = 0.001$) groups (D^2-Mahalanobis = 3.2 and 5.2, respectively).

Although CAN 1 and CAN 2 represent the interactions among the five variables considered, according to the raw canonical coefficients (RCC), the separation between the ruminal acidosis classes along the CAN 1 axis, which was particularly evident between N and A, appeared to be strongly related to β-HB (RCC = 7.8) and glucose (RCC = 1.6). The difference in these variables between the animals experiencing acute ruminal acidosis compared with the others was associated with the higher energy status of the animals fed high grain diets. The separation between the acidosis classes along the CAN 2 axis, which was higher between the A and S heifers, appeared to be related to βHB (RCC = 7.9) and MPV (RCC = 1.5), which could represent an increase in platelet formation due to the onset of ruminal lesions. Further research is needed to confirm this hypothesis.

Conclusions

Many of the blood variables that were investigated showed significant differences between the three diets, although as few as five of them (HGB, MPV, β-HB, glucose and RHb) were sufficient to obtain a canonical structure (CDA). CDA appeared to significantly discriminate between the animals with a physiological ruminal status, SARA or acute ruminal acidosis. Despite these promising results regarding the use of plasma variables to evaluate the severity of short-term ruminal acidosis, additional studies are necessary to confirm the reliability of these discriminant functions during long periods of acidosis both in beef and dairy cattle.

Methods

The experimental protocol was approved by the Animal Ethics Committee of the University of Padova, Italy (CEASA, approval number 88/2011) according to the national laws on the Ethics of Animal Experimentation.

Animals and experimental design

Six crossbred Valdostana x Belgian Blue non-pregnant heifers with an average body weight (BW) of 334±14 kg were used. The animals were kept in loose housing conditions in an 88 square meter pen with a roof and natural ventilation equipped with six feeding stations and two waterers. The straw bedding was replaced daily, but not during the experimental periods to prevent the animals from feeding on it. The BW was measured at the beginning and

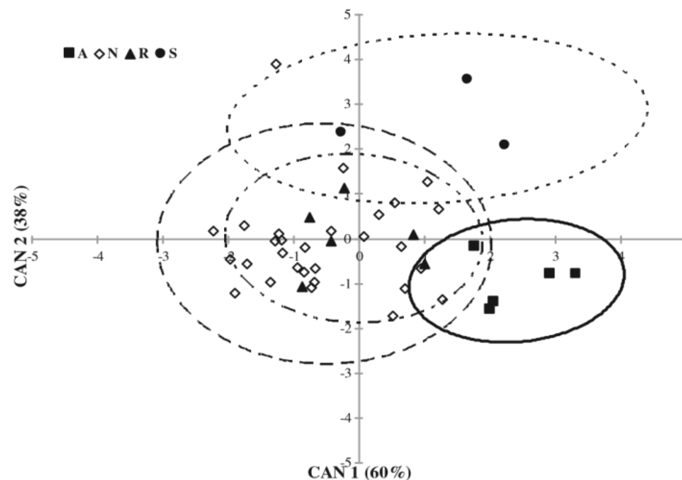

Figure 4 Canonical discriminant analysis scattergram of the four classes of ruminal acidosis. The axes (CAN 1 = 60% and CAN 2 = 38%) account for 98% of the total variability of the measured variables. Ninety-five per cent ellipses are drawn around each centroid of groupings. A = Acute ruminal acidosis (ellipse with a —— line); N = normal acidosis conditions (ellipse with —— line); R = Risk of SARA (ellipse with a - · - · - line); S = SARA (ellipse with a - - - - line). HS diet led to 4 episodes of acute ruminal acidosis in the first period, 1 episode of risk of acidosis in the second period and 1 episode of SARA in the third. MS diet caused 1 episode of acute acidosis, 2 episodes of risk of acidosis and SARA in the first period, whereas it led to 2 episodes of risk of acidosis in the third period. CT diet led to only 1 episode of risk of acidosis in the first period.

the end of the trial. All of the heifers were examined at the beginning of each study period to evaluate their health status

Before the beginning of the trial, the animals were allowed 15 days to adapt to the pen and the CT diet. Each experimental period lasted 5 days and was alternated with a rest period of two weeks during which the animals were fed a CT diet ad libitum and samples were not collected. A 3 x 3 Latin square arrangement of treatments with 3-week experimental periods was used, and the heifers (n=6) were randomly assigned to the three dietary treatments according to the schedule reported in Table 3.

Dietary treatments

Heifers were offered one of three diets characterised by the following different starch levels (Table 4): HS to induce acute ruminal acidosis, MS for SARA or low starch as CT. A similar acidosis challenge model was previously suggested by other authors [18]. The animals were individually restricted and fed three times a day at 0800, 1200 and 1800 h. Water was continuously provided. At each meal, heifers were allowed to feed for approximately 1.5 h until each of the animals had stopped eating for at least 10 min. The residual feed was removed until the next meal. Heifers were fed 10 kg of their ration at each meal, and the feed that was not consumed was removed and weighed.

Acidosis challenge model

Each experimental period was preceded by 3 baseline days (pre-challenge days d-3, -2 and −1) in which the heifers had access to the CT TMR three times per day. On the day before the challenge (restricted feeding day, d0), the feed was restricted to two meals (0800 and 1200 h) with a consequent reduction of DMI (2.8 kg on average). On d1, the HS, MS and CT diets were fed to induce acute acidosis or subacute acidosis or to maintain the physiological ruminal pH, respectively. On the following three days (d2, d3 and d4), all of the animals were fed the CT TMR three times per day.

Feed intake and feed analyses

The weight of the feed offered and refused was recorded at each meal, and the total daily DMI was calculated as the sum of the amount ingested during the daily meals. The diets were sampled twice for each experimental week and analysed for chemical composition [19,20].

Table 3 Treatment sequence applied to the heifers throughout the periods

Period	Treatment[1] CT	MS	HS
1	H1, H2	H3, H4	H5, H6
2	H5, H6	H1, H2	H3, H4
3	H3, H4	H5, H6	H1, H2

[1] CT = control; MS = medium starch; HS = high starch.
H1-H6 = heifers used in the trial.

Table 4 Formulation and composition of diets

Item	Treatment[1]		
	CT	MS	HS
Ingredients, % DM			
Permanent meadow 1st crop	29.0	19.1	14.5
Dehydrated alfalfa hay	16.4	10.6	7.6
Soybean-based blend[2]	15.7	10.8	7.9
Dry beet pulp	6.3	4.3	3.2
Cereal mix[3]	25.6	16.1	13.0
Crushed linseed	4.5	3.1	2.0
Molasses	0.3	0.1	0.1
Vitamin and mineral mix	2.1	1.5	1.3
Maize meal (0.5 mm)	0.0	34.4	50.4
Diet Composition			
DM, %	89.1	87.6	87.8
Crude protein, % DM	16.4	14.3	13.2
Ether extract, % DM	4.5	4.2	4.2
Crude ash, % DM	8.7	6.2	5.0
NDF, % DM	33.0	26.3	20.9
Starch, % DM	17.3	33.4	42.8
Net energy for lactation, MJ/kg DM	6.91	7.66	8.00

[1] CT = control; MS = medium starch; HS = high starch.
[2] 58% soybean meal and 42% extruded de-hulled soybean expeller.
[3] 70% maize meal and 30% barley meal.

Ruminal pH

The ruminal pH was continuously measured in all of the heifers during the entire trial using KB1001 wireless sensors (Kahne Limited, Auckland, New Zealand). Ruminal pH readings were collected every 10 min as suggested by other authors [21]. Fifteen days before commencement of the trial, the sensors were calibrated and delivered per os in the rumen using a sensor release device provided by the manufacturer. To verify the reliability of the pH values recorded by the sensors, ruminal fluid samples were collected from each heifer on the fourth day of each experimental period (d3) by rumenocentesis. The pH was immediately measured using a portable pH meter (Piccolo, Hanna Instruments, Villafranca Padovana, Italy) and compared with the values recorded by the sensors. Rumenocentesis was performed 4 hours after TMR distribution at 1200 h using a 13G, 105-mm needle [22,23].

The pH data from the sensors in each animal were summarised daily as the nadir pH, the maximum pH and the mean pH. The amount of time per day that the pH was below three ruminal pH thresholds (pH < 5.0; $5.0 \leq$ pH < 5.5 and $5.5 \leq$ pH < 5.8) was determined for each heifer during the three experimental periods. Although several rumen pH thresholds have been used to define acute ruminal acidosis and SARA [1,4,7,24], these threshold values were selected because pH < 5.0 leads to the destruction of both cellulolytic and lactate-using bacteria and protozoa and severely damages the rumen mucosa [5,25], pH < 5.5 is detrimental to the ruminal epithelium and VFA absorption [18,26] and pH < 5.8 is harmful to ruminal cellulolytic bacteria [18,27,28].

Blood collection and analysis

Blood samples (20 mL) from the jugular vein were collected in lithium-heparin and K3 EDTA tubes (Vacuette, Greiner Bio-One, Kremsmuenster, Austria) from each animal at 0800 h on each experimental day immediately before the meal. The blood from the K3 EDTA tubes and one subsample of lithium-heparin-preserved blood was refrigerated (4°C) and analysed within 1 h for a complete blood cell count and blood gas analysis, respectively. The other subsamples were immediately centrifuged (1,500 g, 15 min, 4°C) for plasma separation and the plasma was preserved at −80°C until analysis. The complete blood cell count with leukocyte formula was performed using an automated cell counter (Cell Dyn 3500, Abbott Laboratories, Abbott Park, Illinois, USA). Blood gas analysis was performed in a calibrated blood gas analyser (Synthesis 15, IL Instrumentation Laboratory SpA, Milano, Italy) to determine the following variables: pCO_2, pO_2, O_2Hb and RHb. The HCO_3^- level and sO_2m were calculated. Measurements were performed as recommended by the National Committee of Blood Laboratory Standards [29]. The plasma was analysed for the following haematological variables: glucose, CHOL, nonesterified fatty acids (NEFA), β-HB, AST, γGT and LBP. With the exception of LBP, the haematological variables were measured with reagents supplied by Roche Diagnostics and Randox Laboratories Ltd. (NEFA and β-HB) for the Roche Cobas C501 automatic analyser

(Roche Diagnostics, Indianapolis, IN, USA). The concentration of LPS in the plasma was determined by a chromogenic Limulus amoebocyte lysate (LAL) end-point assay (QCL-1000, Lonza Group Ltd. Basel, Switzerland) [30]. The plasma concentrations of LBP were measured [30] using a commercially available kit (HK503, HyCult Biotechnology, Uden, Netherlands). Samples were analysed in duplicate.

Statistical analysis

The normality of the sample distribution was assessed using the Shapiro-Wilk test (PROC UNIVARIATE). The DMI, ruminal pH, blood gas analysis, plasma haematological profile and LBP data were analysed using a mixed procedure with a CS (compound symmetry) structure. The linear model is as follows:

$$Y_{ijkm} = \mu + T_i + P_j + D_k + h_l + TP_{ij} + TD_{ik} + PD_{jk} + TPD_{ijk} + \varepsilon_{ijklm}$$

where μ is the overall mean; T_i is the fixed effect of the dietary treatment with 3 levels: CT, MS, and HS; P_j is the fixed effect of the period with 3 levels; D_k is the fixed effect of the day with three levels: d1, d2 and d3 (one, two and three days after the restricted feeding day); h_l is the random effect of the heifer (2 heifers per T_i); TP_{ij}, TD_{ik} and PD_{jk} are the interactions between the fixed effects; TPD_{ijk} is the interaction between the effects of the dietary treatments, period and day; and ε_{ijklm} is the random residual ~N (0, σ^2_e). Day was considered a repeated measure. If a significant F test was detected ($P < 0.05$) , the treatment means (LSmeans) of the T_i and P_j were compared using the probability of differences (PDIFF) option and the Bonferroni adjustment test. The DMI data on d1 were also evaluated according to a linear random model that included the fixed effects of dietary treatment and period (repeated measures) along their interaction, the random effect of heifers and the random residual. Moreover, a regression coefficient between the rumenocentesis (covariate) and the boluses ruminal pH (data detected only in d3) was determined using the same mixed model.

The average amount of time for each heifer with a pH below the three established pH thresholds (pH < 5.0; 5.0 ≤ pH < 5.5 and 5.5 ≤ pH < 5.8) were not normally distributed (W-values < 0.90), even following transformation. These data were tested using the non-parametric KruskalWallis criteria (PROC NPAR1WAY) to discriminate between the dietary treatments, periods and their interactions (PDIFF Bonferroni adjusted).

Stepwise (PROC STEPDISC) forward canonical discriminant analyses (CDA, PROC CANDISC) were performed separately on the plasma gas and metabolites data (independent variables) to discriminate between the four classes of ruminal acidosis (N, normal ruminal conditions, pH > 5.8; R, risk of ruminal acidosis, 5.5 < pH ≤ 5.8 and 5.0 < pH ≤ 5.5 for less than 4 h; S, subacute ruminal acidosis, 5.0 < pH ≤ 5.5 for at least 4 h; and A, acute ruminal acidosis, pH ≤ 5.0) [25,31,32]. The plasma variables that contributed the most to the discrimination of the ruminal acidosis classes were selected based on the F values ($P < 0.15$) as criterion for inclusion in the stepwise analyses. Wilks' λ and the associated F approximation were used to test the significance and estimate the weight of each plasma variable in CDA. Based on the resulting plasma profile (5 variables reported in Table 3), the squared Mahalanobis distances were calculated to assess the proximity between the rumen acidosis statuses in the predefined classes.

All of the statistical analyses were performed using SAS software (2008; release 9.2).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GM, IA and MM designed the feeding trial which was conducted by RDN and MG, whereas AB and A-LS performed the chemical analyses. GM and SS analysed and interpreted the data, and drafted the article. All authors provide editorial content and have read and approved the final manuscript.

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CHAPTER 4

Blood parameters modification at different ruminal acidosis conditions

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Blood Parameters Modification at Different Ruminal Acidosis Conditions

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Summary

This study evaluated the reliability of various blood parameters to assess the ruminal acidosis in cattle. Six whole heifers were fed three experimental rations in a 3 x 3 Latin square design. The diets had different starch levels: high (HS), medium (MS) or low (CT). Ruminal pH values were continuously measured using wireless sensors. To evaluate the severity of ruminal acidosis, the amount of time per day that the pH was below 5.8, 5.5 and 5.0 was recorded. Blood samples were analyzed for complete blood count, venous blood gas and biochemical profile at 8:00 and 12:00 h. The data were analyzed according to a mixed model. Feeding on CT, MS and HS led to significant differences in DMI (7.7 vs. 6.9 vs. 5.1 kg/d; $P < 0.01$) which modified the amount of time per day that the pH was below 5.0 (0 vs. 12 vs. 92 min; $P < 0.10$). Feeding MS and HS diets led to inflammation as indicated by the significant increment of white blood cells when compared to the CT ones and to blood concentration due to the osmotic pressure at ruminal level. Furthermore a significant decrease of bicarbonate level, CO_2 partial pressure and oxyhemoglobin was observed as consequence of the activation of metabolic processes aimed to prevent metabolic acidosis. No differences were observed on blood sampling time, suggesting that one daily blood sample was enough to evaluate the metabolic variations related to ruminal acidosis.

Key words

dairy cattle, starch level, ruminal acidosis, blood parameters

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Aim

Ruminal acidosis is a metabolic disease in cattle fed high starchy concentrate diets and it has been defined as a disorder associated with low ruminal pH (Plaizier et al., 2009). The drop of ruminal pH is not only influenced by the amount of starch ingested, but also by the starch source (e.g., maize, wheat or barley) and its particle size (De Nardi et al., 2013). Although clinical signs are not always obvious, ruminal acidosis affects dry matter intake (DMI) and productions, can cause laminitis and other disorders resulting in substantial economic losses in dairy farming (Corato et al., 2005; Calsamiglia et al., 2012). Although several parameters were investigated to help in the diagnosis of ruminal acidosis (Gozho et al., 2007; Enemark et al., 2008; Marchesini et al., 2011) only few authors have extensively investigated the effect of ruminal acidosis and sampling time on blood variables (Brown et al., 2000; Marchesini et al., 2013).

The aims of this study were to identify the blood variables change in heifers affected by different levels of ruminal acidosis and to evaluate the variation of these parameters at different sampling times.

Materials and methods

Animals and Experimental Design - According to a 3 x 3 Latin square arrangement, six crossbred Valdostana x Belgian Blue non-pregnant heifers were randomly assigned to 1 of 3 dietary treatments. Each experimental period lasted 5 days and it was followed by a 2 weeks washout period during which the animals were fed the control diet (CT) *ad libitum*.

Dietary treatment - During the experimental period, heifers received one of three total mixed rations (TMR) characterized by different starch levels (% DM): control (CT) as low starch diet (17.3%), medium starch (MS) for SARA (33.4%) and high starch (HS) to induce acute ruminal acidosis (42.8%). The animals were individually restricted and fed three times a day at 8:00, 12:00 and 18.00 h. Heifers were offered 10 kg of their ration at each meal, and the feed that was not consumed was removed and weighed.

Acidosis Challenge Model - Each experimental period was preceded by 3 baseline days (pre-challenge days d-3, -2, -1) in which the heifers had access to the CT TMR three times a day. On the day before the challenge (restricted feeding day, d0), the feed was restricted to two meals a day (8:00 and 12:00 h) with a consequent reduction of DMI. On d1, the HS, MS and CT diets were fed to induce acute acidosis or subacute acidosis or to maintain the physiological ruminal pH, respectively. On the following three days (d2, d3 and d4), all the animals were fed the CT diet three times a day.

Ruminal pH - The ruminal pH of each heifer was measured every 10 min during the entire trial using KB1001 wireless sensors (Kahne Limited, Auckland, New Zealand). As reported by other authors (Dohme et al., 2008; Marchesini et al., 2013), to measure the severity of ruminal acidosis, the amount of time per day that the pH was below three ruminal pH thresholds (pH < 5.0; $5.0 \leq$ pH < 5.5 and $5.5 \leq$ pH < 5.8) was determined for each heifer.

Blood collection and analysis - Blood samples (20 mL) were taken from the jugular vein from each animal at 8:00 and 12:00 h on each experimental day immediately before the meal. The blood was analyzed for complete blood cell count (Cell Dyn 3500, Abbott Laboratories. Abbott Park, Illinois, USA), blood gas analysis (Synthesis 15, IL Instrumentation Laboratory SpA, Milano, Italy), urea and glucose (Roche Diagnostics, Indianapolis, IN, USA).

Statistical Analysis - Sample distribution normality was assessed with the Shapiro-Wilk test. Those variables that showed $W < 0.95$ were log-transformed (natural logarithm) to meet parametric assumptions. However, data of normalized variables are presented in the results section as raw LSmeans. The associated P-values were calculated from the transformed data with the following model. The blood pH, count, gas analysis, and plasma hematological profile data were analyzed using a mixed procedure with a CS (compound symmetry) structure. The linear model included fixed effect of dietary treatment, period, day, daily time, and their interactions. Heifer was considered a random effect and day a repeated measure. In the case of DMI, data were analyzed with the same model but without the daily time effect and its interactions. The degrees of freedom of D effect were used in the two orthogonal contrasts: *D1*, CT vs. (MS and HS)/2; *D2*, MS vs. HS. The average amount of time for each heifer with a pH below the three established pH thresholds (< 5.0; between 5.0 and 5.5; between 5.5 and 5.8) were not normally distributed, thus they were tested using the non-parametric Kruskal-Wallis criteria to discriminate among the dietary treatments. Significance was declared at $P < 0.05$, however a trend was considered to exist if $0.05 < P < 0.10$. All of the statistical analyses were performed using SAS (2008; release 9.2).

Results and discussion

DMI was significantly affected by the treatment resulting the highest in CT-fed heifers (7.7, 6.9 and 5.1 kg/d DM; $P < 0.01$ both in *D1* and *D2*). Figure 1 shows the ruminal pH drop caused by the rapid fermentation due to both the high amount of starch ingested and the small (0.5 mm) mean maize particle size (Plaizier et al., 2009). As expected, the ruminal pH of the animals fed the CT diet tended to spend the lowest ($P < 0.10$) time between 5.0 and 5.5 and never registered values lower than 5.0 (Figure 1).

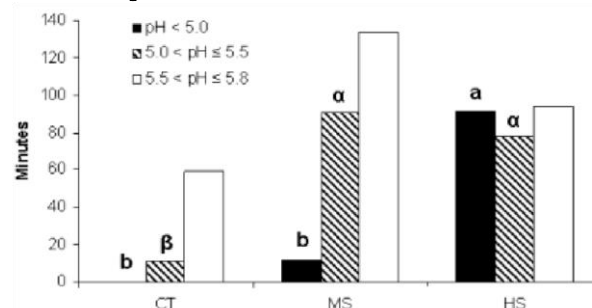

Figure 1. Mean time spent daily below three ruminal pH thresholds ([a, b] Means of time per day below pH 5.0 with different superscripts are different ($P < 0.05$); [α, β] Means of time between pH 5.0 and 5.5 with different superscripts are different ($P < 0.10$))

Table 1. Effect of dietary treatment (D, *n* = 18) and daily time (T, *n* = 36) on blood pH, count, gas and hematological profile

	Dietary treatment						SEM	Probability		
	CT		MS		HS			*D1*	*D2*	T
Daily Time	8	12	8	12	8	12				
pH	7.39	7.39	7.39	7.39	7.39	7.39	0.004	ns	ns	ns
Red blood cells (10^{12}/L)	8.0	7.9	7.6	7.7	8.2	8.0	0.16	ns	*	ns
White blood cells (10^{9}/L)	8.5	9.0	9.8	10.1	9.5	9.7	0.42	**	ns	ns
[§]Neutrophils (10^{9}/L)	2.9	3.1	3.8	4.0	3.8	3.9	0.28	**	ns	ns
Lymphocytes, (10^{9}/L)	4.6	4.8	5.0	5.2	4.8	4.9	0.34	ns	ns	ns
Monocytes (10^{9}/L)	0.84	0.93	0.82	0.82	0.72	0.75	0.068	**	*	ns
Basophils (10^{9}/L)	0.09	0.10	0.08	0.09	0.07	0.07	0.010	*	ns	ns
[§]Eosinophils (10^{9}/L)	0.04	0.04	0.06	0.06	0.13	0.11	0.028	†	†	ns
Hemoglobin (g/dL)	11.1	11.0	10.7	10.8	11.4	11.2	0.16	ns	*	ns
Hematocrit (%)	33.8	32.9	32.8	32.7	34.1	33.3	0.51	ns	*	ns
Platelets (K/μL)	503	519	477	516	601	607	42.8	†	**	ns
pCO_2 (mmHg)	52.0	51.0	50.4	50.8	50.4	49.6	0.42	*	ns	ns
pO_2 (mmHg)	62.1	61.1	71.3	67.8	67.4	64.6	4.26	ns	ns	ns
HCO_3^- (mmol/L)	32.1	31.4	31.2	31.0	30.6	30.3	0.27	**	†	ns
Oxyhemoglobin (%)	87.2	85.5	87.7	88.1	85.4	83.6	1.24	ns	*	ns
Reduced hemoglobin (%)	10.9	11.8	11.1	10.2	13.7	13.7	0.92	ns	*	ns
sO_2m (%)	89.4	88.3	90.0	90.9	87.0	85.8	1.12	ns	*	ns
Urea (mmol/L)	5.32	5.57	4.28	4.24	3.98	3.93	0.122	**	**	ns
Glucose (mmol/L)	4.34	4.34	4.37	4.41	4.32	4.36	0.08	ns	ns	ns

CT, control diet; MS, medium starch diet; HS, high starch diet. *D1*: orthogonal contrast CT *vs* (MS + HS)/2; *D2*: orthogonal contrast MS *vs* HS. The interaction DxT was never significant. **: $P < 0.01$; *: $P < 0.05$; †: $P < 0.10$. [§]Data are presented as raw LSmeans, and the associated P-values are given by statistical analysis of the log-transformed data. pCO_2, partial pressure of CO_2; pO_2, partial pressure of O_2; HCO_3^-, bicarbonate level; sO_2m, measured oxygen saturation.

Compared to CT, MS and HS treatments led to an increase of white blood cells (WBC) and platelets (Table 1). Among WBC, both neutrophils and eosinophils increased, whereas monocytes and basophils decreased. Dong et al. (2011) reported that the early hours following grain engorgement are characterized by the rapid growth of Gram-negative bacteria, which undergo cell lysis and lipopolysaccharides (LPS) release following a reduction in the ruminal pH. The translocation of LPS from the digestive tract to the bloodstream leads to the activation of the systemic immune response that is responsible of the increase of neutrophils and eosinophils. The increase of platelets might represent the response to the onset of ruminal lesions consequent to ruminal acidosis (Steele et al., 2009).

The drop in HCO_3^- level in the MS and HS diet is part of the buffering mechanism to contrast the incoming of metabolic acidosis as a result of ruminal acidosis (González et al., 2012), whereas the reduction in the partial pressure of carbon dioxide (pCO_2) was due to the compensation of the metabolic acid– base imbalance by the respiratory tract, which lowers the pCO_2 to prevent the drop in blood pH through increased ventilation (Dehkordi and Dehkordi, 2011). Significantly lower HCO_3^- and higher platelets values were observed in the HS treatment when compared to the MS one (Table 1), because in the former the ruminal acidosis resulted more severe (Figure 1). The HS diet led also to significantly higher concentrations of red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT), due to the high ruminal osmotic pressure that pulls fluid from plasma into the rumen and concentrates the blood components during ruminal acidosis. Moreover, during the HS treatment, excess organic acids that accumulated in the rumen were absorbed into the bloodstream at the risk of overwhelming the bicarbonate buffering system (González et al., 2012). This caused a shift in

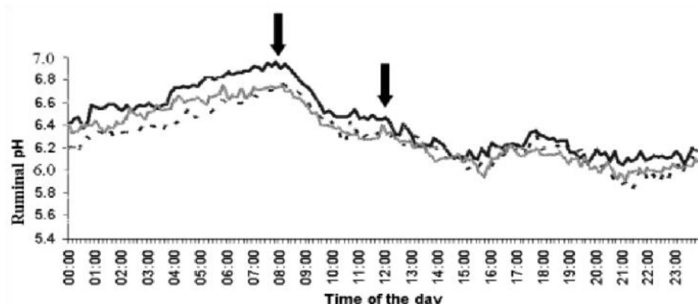

Figure 2. Mean ruminal pH trend at the time of blood collection (CT = control, black continuous; MS = medium starch, black dashed; HS = high starch, gray continuous. Arrows = time of blood collection and feeding (8:00 and 12:00 h)

the oxyhaemoglobin (O_2Hb) dissociation curve (Jones, 2010) and the red blood cells released oxygen to the tissues more readily, which increased the reduced hemoglobin (RHb) and lowered the O_2Hb and the measured oxygen saturation (sO_2m). The urea levels decreased significantly from CT to HS, in line with the crude protein intake.

Sampling time had no effect on any considered blood variables (Table 1). This result could be explained by the fact that the second blood sampling occurred 4 h after the main meal of the day and, as reported in Figure 2, at 12:00 h the ruminal pH showed a reduction of only 0.4 units and had not reached its lowest value yet. The time interval between the main daily meal and the moment in which the ruminal pH reaches its lowest value (nadir pH) is variable and it is related to the feed distribution management. In this study the nadir pH was reached at 21:00 h, 13 h after the main daily meal.

Conclusions

The severity of ruminal acidosis, measured as the amount of time spent daily below three pH thresholds, affected many of the blood variables that are the expression both of the inflammation and the osmotic pressure at ruminal level and of the activation of metabolic processes aimed to prevent the metabolic acidosis. Variables like RBC, WBC, HCT, platelets, pCO2, HCO_3^-, O_2Hb and sO_2m should be taken into account to investigate on ruminal acidosis in cattle, even though additional studies are necessary to confirm their reliability. The blood sampling time did not affect the results suggesting that one blood daily sample is enough to identify the metabolic condition related to the ruminal acidosis.

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CHAPTER 5

Effect of feeding fine maize particles on the reticular pH, milk yield and composition of dairy cows

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Effect of feeding fine maize particles on the reticular pH, milk yield and composition of dairy cows

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Short title: Effect of grain size on reticular pH and milk production

Summary

The particle size of cereal grains has been found to modulate the rate of passage from the rumen and the digestibility of starch and neutral detergent fibre (NDF), but few studies have examined its impact on reticular pH. The aim of this study was to evaluate the effect of feeding finely ground maize on the risk of ruminal acidosis, milk yield and composition. Twelve Holstein Friesian cows were assigned to one of two experimental groups and fed according to a cross-over design (2 diets x 2 periods). Diets were isoenergetic, isonitrogenous and were characterized by the same level of NDF and ADF, differing only in the maize particle size. In the control diet (Ct), the maize meal was ground to 1.0 mm, whereas in the experimental diet, it was finely ground (Fg) to 0.5 mm. The pH and temperature of the reticulum were continuously measured in 8 cows throughout the trial using indwelling wireless sensors. Dry matter intake was higher in cows offered Fg diet than in Ct (19.0 vs. 20.3 kg/d; $p = 0.067$). However, the milk yield and the 3.5% FCM did not show any differences between the diets. Fg led to an increase of milk casein (2.48 vs. 2.57%; $p = 0.035$) and crude protein (3.18 vs. 3.31%; $p = 0.021$). Similarly, starch digestibility increased in animals offered Fg diet vs. Ct (0.94 vs. 0.98; $p = 0.078$). Among the reticular parameters, the Fg-fed cows spent a significantly higher time below the 5.5 pH threshold (15 vs. 61 min/d; $p = 0.047$) and had an average daily variation of reticular pH characterised by a lower nadir pH (5.95 vs. 5.72; $p < 0.001$) and a higher pH range (0.79 vs. 0.94; $p = 0.003$). The grain particle size affected the risk of the onset of ruminal acidosis, and therefore, it should be carefully considered when formulating rations.

Keywords: Maize particle size, reticular pH, SARA, milk composition, dairy cow

Introduction

The physical characteristics of feed, with particular regard to fibre particle size and density, have been extensively studied and reported to influence the ruminal fermentations, feed efficiency and milk composition in dairy cows. The reduction of fibre particle length is known to decrease the mastication and saliva production (Kononoff et al., 2003 b; Beauchemin and Yang, 2005), resulting in reduction of ruminal pH (Dohme et al., 2007). However, reduced fibre particle length also influenced the cow sorting behaviour (Marchesini et al., 2011) and it was found to affect dry matter (DM) total tract digestibility (Tafaj et al., 2007; Maulfair et al., 2011). Manipulating the particle size of cereal grains has been found to affect their rate of passage from the rumen (Knowlton et al., 1998) and the starch and neutral detergent fibre (NDF) digestion at ruminal level (Callison et al., 2001; Rémond et al., 2004); in addition it resulted also in the modification of the total tract starch digestibility and the protein content of milk (Cao et al., 2008). These effects depend on the cereal type (i.e., species and vitreousness of the endosperm) and on the diameter of the particles (Knowlton et al., 1998; Rémond et al., 2004). Ground grain has a wider surface available for microbial attack, which could lead to fast and complete ruminal degradation, reducing faecal starch excretion (Callison et al., 2001; Rémond et al., 2004) and increasing the efficiency of starch utilisation (Corato et al., 2005). In addition to the positive effects of a reduction in grain particle size, a raising rate of starch fermentation could lead to an increase in the level of volatile fatty acids (VFA), resulting in a reduction of ruminal pH (Kleen et al., 2003) and an increase in the risk of subacute ruminal acidosis (SARA), defined as the condition in which rumen pH is depress for prolonged periods each day (Plaizier et al., 2009; Marchesini et al., 2013). This metabolic disorder has been shown to cause consistent economic losses in dairy farming (Enemark, 2008) and to be responsible for the onset of different pathologies, such as rumenitis, parakeratosis, metabolic acidosis and laminitis (Enemark, 2008; Steele et al., 2009). Although other authors have investigated the effects of maize particle size on ruminal pH (Rémond et al., 2004; Cao et al., 2008), the pH was not continuously measured in their studies, leading to a lower sensitivity in the detection of SARA when compared to the continuous acquisition of data (Dado and Hallen, 1993). The aim of the present study was to determine whether feeding finely ground maize could cause an increased risk of SARA in high producing dairy cows by continuously recording reticular pH and temperature. In addition, the effects of

maize particle size on dry matter intake (DMI), feed total tract apparent digestibility, milk yield and milk composition were also investigated.

Materials and methods

Dietary treatment, experimental design and animals

During a trial lasting 42 days, twelve Holstein Friesian cows were randomly assigned to one of two experimental groups that were balanced for milk yield (34.1±3.6 kg/d), days in milk (DIM, 210±64 d), parity (1.7±0.8), body weight (BW, 651±28 kg) and 1 to 5 scale body condition score (BCS, 2.5±0.3). Animals were allowed 7 days for dietary and pen adjustment. The cows were assigned to two experimental groups and were exposed to one of two dietary treatments in a 2 × 2 cross-over design (2 diets x 2 periods) over a period of 21 days (14 days of an adjustment phase followed by 7 days of data collection). As reported in Table 1, the treatments consisted of two isoenergetic and isonitrogenous total mixed rations (TMRs) that were characterized by similar NDF and ADF content, differing only in the maize meal particle size. In the Ct diet, the maize meal was ground to 1.0 mm, whereas, in the experimental diet, the maize meal was finely ground (Fg) to 0.5 mm. The grinding procedures were made by the feed supplier using a cylindrical running mill. The cows were housed in a free stall in two pens. Feeding time was at 8:00 and 15:00 hours whilst milking was conducted at 5:00 and 17:00 hours with an automated milking plant.

Reticular pH and temperature

The pH and temperature (T) of the reticulum were continuously measured in four cows for each group during the entire trial using wireless sensors (smaXtec Animal Care, Graz, Austria). Seven days before commencement of the trial, the sensors were calibrated as recommended by the producer and delivered in the reticulum using a balling gun. Reticular pH readings were collected every 10 min as suggested by McLaughlin et al. (2009).

The pH data from the sensors in each animal were summarised daily as the nadir pH, the maximum pH and the mean pH. The amount of time per day that the pH met one of three pH thresholds ($pH < 5.5$, $5.5 \leq pH < 5.8$ and $5.8 \leq pH < 6.3$) was determined for each cow equipped with the sensor during each experimental week. Although several rumen pH thresholds have been used to define SARA (Dohme et al., 2008; Plaizier et al., 2009), these threshold values were selected because $pH < 5.5$ is detrimental to the ruminal epithelium and VFA absorption (Dohme

et al., 2008), pH < 5.8 is harmful to ruminal cellulolytic bacteria (Dohme et al., 2008) and pH < 6.3 is the threshold proposed by Sato et al. (2012) for SARA determination from reticular fluid, given that the reticular pH is slightly higher than the ruminal pH due to mixing and dilution with saliva (Duffield et al. 2004). Temperature (T) data were summarised daily as the mean, minimum and maximum values and as the time spent by the animals in three T (°C) thresholds ($39.0 \leq T < 39.2$; $39.2 \leq T < 39.4$ and $T > 39.4$) as suggested by AlZahal et al. (2008).

Samples management and analysis

The mean geometric particle size of maize for both dietary treatments were measured according to ASAE procedures (1995) and was equal to 978 and 511 μm for Ct and Fg, respectively. The BW and BCS were measured at days 0, 21 and 42 of the trial. Feed intake was individually and continuously recorded using an automated feeding control system (Biocontrol A/S, Rakkestad, Norway). The TMRs, orts and supplements were sampled twice per week during the experiment. One subsample of each TMR and ort was taken to measure the particle size distribution using a Penn State Particle Separator (Kononoff et al., 2003 a). The other subsample was stored at −20 °C and used later for chemical analysis. The feed samples were dried at 60 °C for 48 h, ground to pass a 1-mm screen, and then analysed for dry matter (DM), crude protein (CP), ether extract (EE), crude ash (CA), NDF, ADF and acid insoluble ashes (AIA) according to AOAC (2003) and Van Soest et al. (1991) using a Fibre Analyser (ANKOM/2000, ANKOM Technology, New York, USA). The peNDF was calculated according to Mertens (1997) and Beauchemin and Yang (2005). The starch content (AOAC, 2005) was determined using high-performance liquid chromatography equipped with a LC 9A pump, SIL auto sampler and the RID-10 A model (Shimadzu, Tokyo, Japan); separations were achieved using a 300 × 7.8 mm Aminex HPX-87H column and one pre-column (Bio-Rad, Hercules, CA, USA) at 40 ◦C.

During each experimental period, the individual milk production was recorded at each milking during each experimental week. The daily (half morning and half evening) milk samples from each animal were collected on days 16 and 20 of each period and stored at 4 °C until analysis. The samples were analysed for fat, protein, casein and lactose content by a MilkoScan FT plus infrared analyser (Fossomatic, Foss Electric, Hillerød, Denmark), and the milk urea content was determined using differential pH-metry (EUROCHEM CL 10 plus, Microlab EFA).

The total tract digestibility of DM, NDF and starch was estimated using AIA as an internal marker (Van Keulen and Young, 1977; Galloway et al., 1993). Faecal samples of each animal

were collected from the rectum every 6 hours on two of the days in each experimental period to represent different times of day in a 24-h period. Faecal sample DM was determined after drying. Dried faecal samples were ground to pass a 1-mm screen, pooled by cow and retained for measurements of AIA and starch. Faecal analyses were performed according to the methods described for feed.

Statistical analysis

After verifying the normality and variance homogeneity (PROC UNIVARIATE and Shapiro-Wilk test), a REML mixed model procedure (PROC MIXED) was performed to evaluate the BCS, BW, DMI, total tract starch digestibility, milk composition and reticular pH data. The linear random model included the fixed effects of dietary treatment and period along with their interaction, the random effect of cow and the random residual. The linear model is as follows:

$$Y_{ijkl} = \mu + D_i + P_j + c_k + DP_{ij} + \varepsilon_{ijkl},$$

where μ is the overall mean; D_i is the fixed effect of the dietary treatment with 2 levels: Ct and Fg; P_j is the fixed effect of the period with 2 levels; c_k is the random effect of the dairy cow; DP_{ij} is the interaction between the fixed effects; and ε_{ijkl} is the random residual ~ $N(0, \sigma^2_e)$.

The analysis of the daily average reticular pH data was also carried out using a similar mixed model that included the repeated effect of day (seven levels) and its interactions with the other fixed effects. Because the 3-way interaction (dietary treatment x period x day) was never significant ($p > 0.20$), it was removed from the model, and the reduced model was re-run. LSMeans of the day effects were separated using the PDIFF (probability of difference) option along with a Bonferroni adjustment for multiple comparisons.

According to a 2 × 2 cross-over design, a two-way ANOVA (PROC GLM) was performed on particle distribution and sorting activity for each fraction to test the fixed effects of dietary treatment and period along with their interaction.

The average amount of time spent by each dairy cow with a pH in the three established pH thresholds ($pH < 5.5$, $5.5 \leq pH < 5.8$ and $5.8 \leq pH < 6.3$) were not normally distributed (W-values < 0.90), even after logarithmic transformation. These data were tested using the non-parametric Kruskal-Wallis criteria (PROC NPAR1WAY) to discriminate between the dietary treatments, periods and their interaction. All of the statistical analyses were conducted using SAS (2008).

Results

As expected, diets showed the same distribution of TMR particle lengths because the ground maize stood in the bottom pan and forage and by-products particles were uniform across treatments (Table 2). The dietary treatments had the same sorting level showing a preference for the smallest particles represented by the concentrates (Table 2). DMI was higher ($p = 0.067$) in Fg-fed cows, although the milk yield and the 3.5% FCM did not show any differences between diets (Table 3). BW (647 vs. 652 kg) and BCS (2.58 vs. 2.60) did not differ ($p > 0.10$) between the diets and did not change throughout the experiment. As reported in Table 3, Fg led to an increase of milk casein and total protein, whereas the other milk composition parameters were not affected by diet; moreover, Fg showed higher level of starch digestibility (Table 3). Nor the temperature parameters (Table 3), neither the daily time spent below the temperature thresholds (Figure 1) were affected by dietary treatment. Although the nadir, mean and maximum pH did not show any significant differences between Fg and Ct (Table 3), the Fg-fed cows spent a significantly higher time below the 5.5 pH threshold (15 vs. 61 min/d; $p = 0.047$; Figure 2). The mean daily variation of reticular pH (Figure 3) shows that Fg-fed cows were characterised by a significantly lower daily nadir pH (5.95 vs. 5.72; $p < 0.001$; SEM = 0.057), a higher (0.79 vs. 0.94, $p = 0.003$; SEM = 0.048) daily pH range (maximum pH minus nadir pH) and a lower maximum pH (6.74 vs. 6.66; $p = 0.093$; SEM = 0.047). No interactions between day and dietary treatment or period on the reticular pH were observed. A significant ($p < 0.01$) effect of day was detected for both the maximum reticular pH and pH range, with the highest values on day 5.

Discussion

In the last years, dairy feed suppliers showed an interest in reducing cereal particles size to increase starch digestibility. In Italy, because of the high vitreousness of the maize used, 1-mm ground maize was already added to TMR, but so far there is a lack of knowledge on the effects of maize of even smaller particle size, especially at ruminal level.

The sorting of fine particles at the expense of other fractions was in line with the results of previous studies (Kononoff et al., 2003 b; Marchesini et al., 2011), and it was also consistent with the low water content of the rations. The cows offered the finely ground maize had a greater DMI versus those offered CT, consistent with what reported by Knowlton et al. (1998). However, the increased DMI did not affect milk yield or 3.5% FCM, suggesting that Fg animals had a lower feed efficiency as reported in Table 3. It is generally recognised that the efficiency of metabolisable energy utilisation from starch is greater when the starch is digested in the small

intestine and absorbed as glucose than when starch is fermented in the rumen to VFA (Reynolds, 2006). In this study, it seems that the smaller maize particles of Fg were fermented at the ruminal level at a higher proportion then that of Ct. This hypothesis is confirmed by the lower feed efficiency, the higher milk concentration of casein and total proteins and the lower reticular pH found in the Fg-fed cows. As reported by Cao et al. (2008), smaller maize particles increase the surface area for microbial adhesion, which leads to fast and complete ruminal degradation and to a greater availability of ruminal fermentable energy for microbial protein synthesis, leading to an increase of milk protein percentage. The latter findings could partially justify the apparent lower efficiency in term of 3.5% FCM, indicating a shift of metabolisable energy from lactose and triacylglycerols to *de-novo* protein synthesis. The faster and more complete maize degradation at the ruminal level is supported by the significant evidence that the Fg-fed cows spent more time daily with a reticular pH below 5.5. Therefore, the faster fermentation is the cause of faster VFA and lactic acid production and accumulation in the rumen, which lead to a pH reduction if rumen buffering cannot keep pace with the accumulation of these acids (Plaizier et al., 2009; Aschenbach et al., 2011). The Fg-fed animals spent around an hour per day with a pH below 5.5, showing a higher risk of SARA compared to Ct, despite the starch level of the ration being the same. Although in this study the cows spent less than 4 hours per day below the 5.5 pH, standard to be diagnosed as being affected by SARA (McLaughlin, 2009), it should be taken into account that the pH data refer to the reticulum, where the pH it is usually higher than in the rumen (Sato et al., 2012). The different pattern of pH between diets is in contrast with the results of Cao et al. (2008), who did not find any differences in ruminal pH between ground and cracked maize. The disagreement between the two studies could be linked to the fact that mean ruminal pH is less sensitive than the time spent in predetermined pH thresholds to detect ruminal pH differences. Moreover, cows in this study were subjected to diets characterised by a lower NDF and higher starch content, challenging the rumen buffering capacity the most. The different influence of the two dietary treatments on reticular pH is also confirmed by its mean daily variation. Although the pH of the two diets had a similar fluctuation throughout the experimental weeks, showing a similar bacterial response to the feed distribution, the pH in cows fed the Fg diet was characterised by the lowest daily nadir pH and the highest daily pH range. These differences are the result of the higher and faster fermentation of the finely milled maize by ruminal bacteria that lead to a higher and faster production of VFA and lactic acid (Aschenbach et al., 2011). The effect of the day on the maximum pH and pH range occurred after five days from the beginning

of the experimental week, most likely indicating that the buffering system was able to cope with the altered ruminal conditions at least for 4 days. As a consequence of the higher fermentation at the ruminal level, the Fg diet led only to a slightly higher total tract apparent starch digestibility, as previously reported by other authors (Rémond et al., 2004), although the DM digestibility remained unaffected. Additional effects on total tract apparent digestibility of post-ruminal digestion and fermentation in the hindgut cannot be excluded (Callison et al., 2001; Reynolds, 2006).

Conclusions

Under the same feeding conditions, reducing the maize particle size resulted in a drop of reticular pH, an increase of milk protein content and a slight reduction of feeding efficiency. Based on the results from this study, the grain particle size should be carefully considered during the ration formulation, taking into account the herd health conditions, milk yield and milk composition, to maximise the production and quality without excessively increasing the risk of ruminal acidosis.

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Table 1. Formulation (% DM), composition and physical effective NDF (peNDF) of Ct (maize meal ground to 1.0 mm) and Fg (maize meal ground to 0.5 mm) dietary treatment

	Dietary treatment	
Ingredients (% DM)	Ct	Fg
Maize meal 0.5 mm	0.0	30.3
Maize meal 1.0 mm	30.3	0.0
Dehydrated alfalfa hay	20.2	20.2
Permanent meadow 1st crop	16.2	16.2
Extruded soybean hull	6.1	6.1
Soybean meal	4.0	4.0
Extruded de-hulled soybean expeller	4.0	4.0
Barley meal	4.0	4.0
Sugar beet dry pulps	4.0	4.0
Straw	4.0	4.0
Corn gluten meal	4.0	4.0
Molasses	1.6	1.6
Vitamin and mineral mix	1.6	1.6
Diet composition		
Dry matter (%)	89.4 ± 0.5	89.8 ± 0.5
Crude protein (% DM)	17.1 ± 0.3	16.9 ± 0.2
NDF (% DM)	33.9 ± 0.9	34.0 ± 1.1
ADF (% DM)	18.0 ± 0.4	18.2 ± 0.5
AIA (% DM)	1.1 ± 0.2	1.0 ± 0.3
Ether extract (% DM)	3.0 ± 0.1	3.0 ± 0.1
Crude ash (% DM)	7.0 ± 0.1	7.5 ± 0.2
Starch (% DM)	25.0 ± 0.4	25.1 ± 0.4
peNDF-8 mm* (% DM)	5.5 ± 0.4	5.8 ± 0.8
peNDF-1.18 mm† (% DM)	16.4 ± 0.7	15.0 ± 1.1
Net Energy for lactation (Mcal/kg of DM)	1.48	1.48

* According to Beauchemin and Yang (2005), peNDF-8 mm was calculated as: [(S1 + S2) / 100] x NDF. † According to Mertens (1997), peNDF-1.18 mm was calculated as: [(S1 + S2 + S3) / 100] x NDF.

Table 2. TMR particles length distribution and sorting index (SI) measured by using a Penn State Particle Separator (*n* = 12)

	Dietary treatment		SEM	p-Value
	Ct	Fg		
Sieves distribution* (%)				
S1	1.4	1.0	0.11	0.218
S2	14.9	16.0	1.65	0.718
S3	32.2	27.1	0.66	0.114
B	51.4	55.9	2.20	0.384
Sorting index†				
SI-S1	104.5	90.1	3.18	0.193
SI-S2	74.8	90.0	3.71	0.211
SI-S3	89.9	90.6	2.11	0.847
SI-B	121.5	109.6	2.44	0.182

*Sieves: S1, 19.0 mm; S2, 8.0 mm; S3, 1.18 mm; B, bottom pan.
†Sorting index (> 100, active selection; = 100, no selection; < 100, active avoidance).

Table 3. Effects of the Ct (maize meal ground to 1.0 mm) and Fg (maize meal ground to 0.5 mm) on the dry matter intake (DMI), milk yield, 3.5% fat-corrected milk (FCM), milk composition, reticular pH, reticular temperature (T) and starch digestibility

	Dietary treatment		SEM	p-Value
	Ct	Fg		
DMI (kg/d)	19.0	20.3	0.82	0.067
Milk yield (kg/d)	32.5	32.3	1.27	0.855
3.5% FCM (kg/d)	30.8	30.5	1.25	0.724
Feed efficiency*	1.63	1.52	0.041	0.008
Milk fat (%)	3.22	3.19	0.171	0.739
Milk protein (%)	3.18	3.31	0.058	0.021
Milk casein (%)	2.48	2.57	0.047	0.035
Milk lactose (%)	4.88	4.89	0.069	0.793
Milk urea (mg/dL)	22.4	21.8	1.40	0.728
Nadir reticular pH	5.95	5.72	0.239	0.262
Mean reticular pH	6.34	6.18	0.212	0.352
Max reticular pH	6.74	6.66	0.188	0.582
Lowest reticular T (°C)	35.4	35.4	0.39	0.959
Mean reticular T (°C)	38.9	39.0	0.15	0.401
Highest reticular T (°C)	40.2	40.2	0.16	0.944
DM digestibility	0.70	0.75	0.030	0.347
NDF digestibility	0.50	0.52	0.034	0.508
Starch digestibility	0.94	0.98	0.013	0.078

*Feed efficiency calculated as ratio between 3.5% FCM (kg/d) and DMI (kg/d). The interaction D x P effect was never significant.

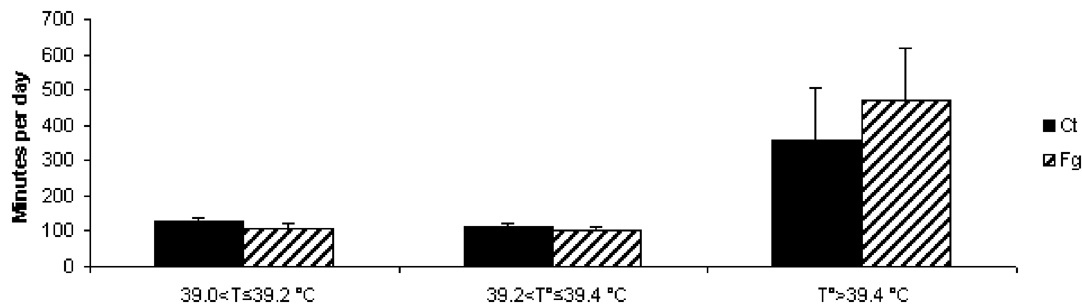

Figure 1. Mean amount of time per day in the reticular temperature (T, °C) thresholds ($39.0 \leq T < 39.2$; $39.2 \leq T < 39.4$ and $T > 39.4$) according to the dietary treatment (Ct = maize meal ground to 1.0 mm; Fg = maize meal ground to 0.5 mm). The difference between diets was never significant ($p > 0.10$). Bar = standard error of mean.

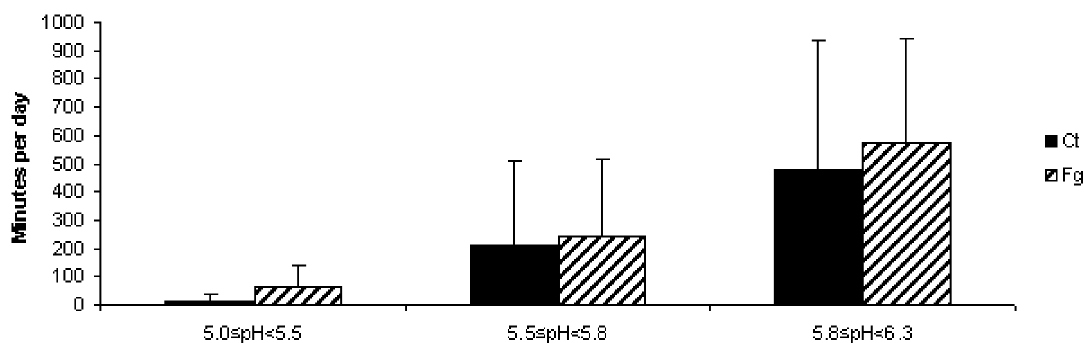

Figure 2. Mean amount of time per day in the reticular pH thresholds ($5.0 \leq pH < 5.5$; $5.5 \leq pH < 5.8$ and $5.8 \leq pH < 6.3$) according to the dietary treatment (Ct = maize meal ground to 1.0 mm; Fg = maize meal ground to 0.5 mm). Cows' reticular pH has never gone below 5.0. According to the non-parametric Kruskal-Wallis test, the difference between diets was significant ($p = 0.04$) for $5.0 \leq pH < 5.5$. Bar = standard deviation.

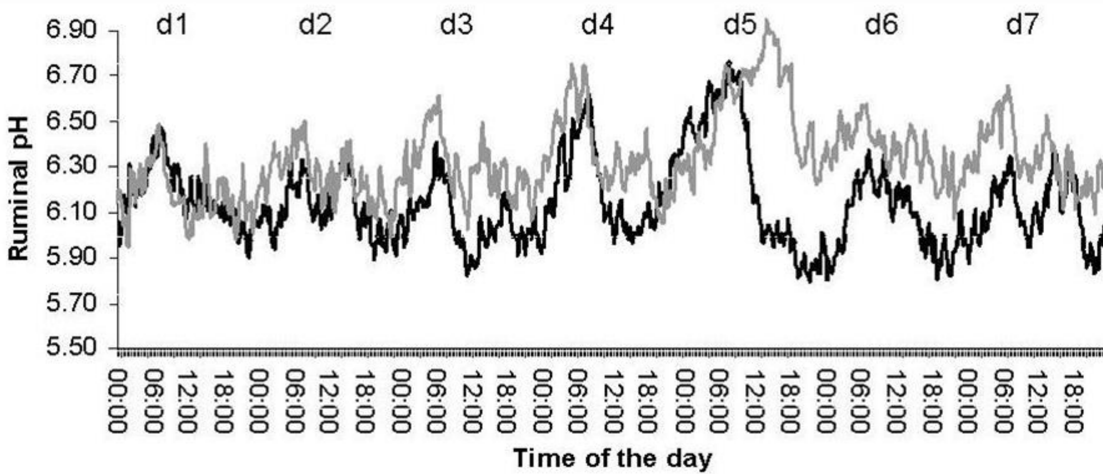

Figure 3. Mean daily (d1-d7) reticular pH variation according to the dietary treatment and the day of the experimental week. Ct = maize meal ground to 1.0 mm, gray continuous; Fg = maize meal ground to 0.5 mm, black continuous. Cows were fed at 08:00 and 15:00 hours; and milked twice at 05:00 and 17:00 hours. Dietary treatment affected nadir reticular pH ($p < 0.001$; SEM = 0.054) and max reticular pH ($p = 0.093$; SEM = 0.047) within each day. Additionally, the daily range (the difference) between max and nadir pH of the two dietary treatments was affected ($p = 0.003$; SEM = 0.048) every day. The interactions among fixed effects dietary treatments and period and the repeated effect of day were never significant ($p > 0.10$). The day effect affected ($p < 0.01$; SEM = 0.083) the maximum pH and the pH range and, in both the variables, the Bonferroni-adjusted LSMeans were: d1 d2, d3, d6, d7 = ^{B}, d4 = ^{AB} and d5 = ^{A}.

CHAPTER 6

Effects of carbohydrase inhibiting compounds on *in vitro* rumen fermentation

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PAPER

Effects of carbohydrase inhibiting compounds on *in vitro* rumen fermentation

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Abstract

Batch culture fermentations with ruminal content were conducted to determine the effects of plant-derived [bilberry extract (BBE), phaseolamin, white mulberry (WMB), common flax] carbohydrase-inhibiting compounds on microbial fermentation. The cultures with these compounds, at two different doses (15 and 150 mg), were compared with both acarbose (ACB) and batch cultures without the addition of any enzyme-inhibiting compounds (Control). Incubations were conducted in triplicate and replicated. The pH, volatile fatty acids, ammonia N, apparent dry matter (DMD) and starch disappearance were measured after 5 and 24 h of incubation. Treatment with ACB, after 5 h, significantly reduced maize meal fermentation, resulting in the highest pH levels (P<0.01), the lowest total VFA concentration (P=0.01) and the lowest DMD (P<0.01). On the opposite, BBE and WMB caused the highest drop in pH, due to the rapid fermentation of their sugar content. Treatment with BBE resulted in an increase in propionate and in an apparently low ammonia N concentration, whilst ACB (150 mg) led to the highest values of acetate (P<0.05) and to a relative high concentration of ammonia N. After 24 h the differences in the fermentation pattern among supplements remained similar to those found after 5 h. In addition, BBE showed an activity against starch degradation, although this effect was concealed by the fermentation of sugars present in that supplement. These results show that some compounds modify the fermentation pattern of the substrate, but further studies are needed to clarify their impact on the complex rumen microbial community.

Introduction

Over the last 10 years of human medical and animal nutrition research, a number of studies have focused on the use of α-amylase and α-glucosidase inhibitors to manage disorders of carbohydrate metabolism, such as type 2 diabetes and ruminal acidosis (Bischoff, 1994, 1995; Martin and Montgomery, 1996; McLaughlin *et al.*, 2009a, 2009b). These supplements have been shown to reduce the degradation of starch and the absorption of glucose in the proximal portions of the small intestine, thereby lowering the post-prandial serum glucose levels (Tarling *et al.*, 2008). Some carbohydrase inhibitors currently in clinical use include acarbose (ACB), a pseudotetrasaccharide extracted from cultures of *Actinomyces* bacteria and miglitol, a pseudomonosaccharide derivative of 1deoxynojirimycin, which inhibits α-glucosidase and α-amylase (Speight and Harmon, 2010) . These synthetic hypoglycaemic agents can cause serious gastrointestinal side effects (Rosenstock *et al.*, 1998; Cheng and Fantus, 2005); therefore, natural compounds are currently being evaluated for their ability to treat diabetes without causing side effects. These natural compounds, including bilberry (*Vaccinium myrtillus*), phaseolamin from the kidney bean (*Phaseolus vulgaris*), seeds of *Linum usitatissimum* and leaves of *Morus alba*, have shown the ability to inhibit α-amylase and α-glucosidase *in vitro* (Mosca *et al.*, 2008; Chu *et al.*, 2011; Sudha *et al.*, 2011). As demonstrated for acarbose by McLaughlin *et al*. (2009a, 2009b), dietary carbohydrase inhibitors can benefit cattle and dairy cows fed high-grain diets by preventing the onset of ruminal acidosis. These inhibitors can slow the fermentation of starch, consequently preventing the rapid drop in ruminal pH, especially in the first 5 h after the meal (Nordlund and Garret, 1994) and precluding the economic losses associated with ruminal acidosis (Krause and Oetzel, 2005). Moreover, these inhibitors can increase the amounts of highly digestible starch that reach the small intestine, where digestion and absorption are most efficient (Harmon and McLeod, 2001; Reynolds, 2006; De Nardi *et al.*, 2014). The aim of this study was to verify the effectiveness of four plant extracts (bilberry, phaseolamin, white mulberry and common flax) and acarbose in reducing the ruminal fermentation of maize meal. Incubations were performed in batch culture after 5 and 24 h and the degree of fermentation was estimated through the measurement of pH, volatile fatty acids (VFA), changes in ammonia N and the apparent disappearance of dry matter (DMD) and starch (STD).

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Supplements

Phaseolamin (PHA) from *Phaseolus vulgaris*, extract from the white mulberry (*Morus alba*) leaf (WMB) and the seed extract from *Linum usitatissimum* or common flax (CFL) were selected for their inhibitory activities against α-amylase and α-glucosidase *in vitro* (Mosca *et al.*, 2008; Sudha *et al.*, 2011) and for their commercial availability. Bilberry (*Vaccinium myrtillus*) extract (BBE) was selected because it is rich in anthocyanins (Chu *et al.*, 2011), which are phenolic compounds that have shown potential for reducing hyperglycaemia in humans (Zunino, 2009) and demonstrated inhibitory activity against α-amylase and α-glucosidase (Johnson *et al.*, 2011). Acarbose was chosen as a positive control because it was found to be effective in reducing ruminal fermentation of cereal grains in both *in vitro* (Speight and Harmon, 2010) and *in vivo* (McLaughlin *et al.*, 2009a, 2009b) studies. All of the extracts were commercially available and were produced by Farmalabor (Farmalabor, Canova di Puglia, Italy), with the exception of CFL, which was obtained

from air-dried flax seeds (Sella Farmaceutici, Schio, Italy). Briefly, the air-dried flax seeds were

finely crushed, powdered and extracted in isopropanol. Isopropanol was added to the flax seeds at a ratio of 1:5 (w/v) and the mixture was incubated overnight in a vessel with a magnetic stirrer (modified from Sudha *et al.*, 2011). The extract was collected, centrifuged, filtered and concentrated *in vacuo* at 35°C for 5 h using an evaporator (Standard EZ-2; Genevac Ltd., Ipswich, UK). Acarbose (Glicobase®) was obtained from the Bayer Corporation (Leverkusen, Germany).

Animals and collection of rumen fluid

Using an oesophageal probe, rumen fluid was collected from 3 steers, which were fed the following total mixed ration once daily (based on wet weight): maize silage (7.0 kg), maize grains (4.1 kg), soybean meal (1.5 kg), dried sugar beets (1.2 kg), wheat straw (0.7 kg) and wheat bran (0.7 kg). The rumen fluid was strained through two layers of cheesecloth, stored at 39°C in a pre-heated thermos and immediately transferred to the laboratory.

Treatments and analyses

The four plant extracts and ACB were added separately to 0.5 g of maize meal (maize grain that was ground through a 2-mm screen) in 50mL polypropylene screw-cap culture tubes. Tubes containing the substrate but no inhibitor were used as the control (Control). A low (15 mg) and high (150 mg) doses were used for each supplement, according to the effective doses found by Speight and Harmon (2010) for ACB and other carbohydrase inhibitors.

Because the most critical period for the prevention of ruminal acidosis corresponds to the first 4-6 h after the feed delivery (Nordlund and Garret, 1994), three tubes (replicates) were tested for each supplement and dose after 5 h of incubation and another three tubes were tested after 24 h. Each tube was filled with 13.4 mL of reduced buffer (McDougall, 1948) and 26.6 mL of strained rumen fluid and incubated at 39°C. To maintain an anaerobic environment, carbon dioxide was added to the tubes immediately before they were sealed. All tubes were incubated in a water bath at 39°C and the experiment was performed twice. At the end of the incubation period, the pH was measured using a portable pH meter (Basic 20; Crison Instruments, Alella, Spain) and 8 mL of each sample was collected for the measurement of VFA and ammonia N. Subsequently, 2 mL of mphosphoric acid (250 g/L) was added to the samples, which were then frozen at -20°C until analysis. After thawing, the samples were centrifuged at 4000×*g* for 30 min at 4°C and the supernatants were filtered using 0.45-m Phenex-RC filters (Phenomenex Srl, Castel Maggiore, Italy).

One subsample of the filtrate was analysed for ammonia N using a SmartChem 200 spectrophotometer (Unity Scientific, Brookfield, CT, USA). For the VFA analysis, a second subsample was injected into an HPLC system complete with an LC 9 A Shimadzu pump, a SIL 10A auto sampler and an RID-model Shimadzu 10A detector (Shimadzu, Kyoto, Japan). Volatile fatty acids separation was performed at 40°C using an Aminex HPX87H column (300×7.8 mm) and one pre-column (Bio-Rad, Hercules, CA, USA). Class VP software was used for data collection and integration. For the complete HPLC analysis of VFA, a 30-min isocratic program was run with 0.025 N H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Peaks of analytes were identified by comparing the retention times of standard mixtures to those of the samples and quantification was based on peak area measurements by an external standard method. The maize meal and supplements were analysed for dry matter (DM; #934.01; AOAC, 2003), crude protein (CP; #976.05; AOAC, 2003), ether extract (EE; #920.29; AOAC, 2003), ash (#942.05; AOAC, 2003), neutral detergent fibre (aNDF), as suggested by Mertens (2002) and starch (#996.11; AOAC, 2000); their composition is reported in Table 1. The aNDF fraction, including residual ash, was determined with α-amylase and sodium sulphite, using an Ankom[220] Fibre Analyser (Ankom Technology, Macedon, NY, USA), whereas starch was determined using high-performance liquid chromatography with a LC 9A pump, a SIL auto sampler and a RID-10 A detector (Shimadzu). Non-fibre carbohydrates (NFC) were calculated as [100-(Ash+CP+EE+aNDF)]. Moreover, to calculate DMD and STD, the DM and starch were measured in the strained and buffered ruminal fluid (before fermentation) and in each sample (5 h or 24 h). Before the analyses each sample was previously dried and ground to pass 0.5 mm. DMD was calculated according to the Wisconsin method, reported by Meyer *et al.* (1971):

DMD=(substrate DM+inoculum DM-residual DM)/(substrate DM)

where substrate is represented by maize meal and supplements, the inoculum is the strained and buffered rumen fluid and the residual is the whole sample (*i.e.* rumen fluid, residual maize meal and residual supplement) after fermentation (5 h and 24 h). The same formula was also applied to calculate STD.

Statistical analysis

The normality of the sample distribution of data was assessed using the Shapiro-Wilk test (PROC UNIVARIATE). The W value of the ammonia N was below 0.95 and the data were therefore log-transformed (natural logarithm) to meet parametric assumptions before the statistical analysis was performed. In a preliminary statistical analysis, the data were submitted to a one-way ANOVA (PROC GLM) that considered the fixed effect incubation time (5 and 24 h). ANOVA was also used to examine the effect of supplement (BBE, PHA, CFL, WMB, ACB), dose (15 and 150 mg), period (1 and 2) and their relative interactions on *in vitro* ruminal pH, VFA and ammonia N concentration, DMD and STD within incubation time (5 and 24 h). LS*means* were compared using the probability of differences option and the Tukey's HSD adjustment test. All the statistical analysis were carried out by using the software SAS (2008).

Table 1. Chemical composition of maize meal and plant-derived supplements.

	Maize meal	Supplements			
		WMB	BBE	PHA	CFL
DM, g/kg	870	920	917	908	1000
Crude protein, g/kg DM	99	19	3	212	2
Ether extract g/kg DM	44	-	4	20	998
Ash, g/kg DM	15	30	3	106	-
aNDF, g/kg DM	121	14	6	30	-
NFC, g/kg DM	721	937	984	632	-
Starch, g/kg DM	707	-	-	-	-

WMB, extract of the white mulberry (*Morus alba*) leaf; BBE, bilberry extract from *Vaccinium myrtillus*; PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; DM, dry matter; CP, crude protein; EE, ether extract; aNDF, neutral detergent fibre; NFC, non-fibre carbohydrates equal to 100-(CP+EE+ashes+aNDF).

Results and discussion

The aim of the present study was to evaluate several plant- and microbial-derived supplements for their inhibitory capacity towards carbohydrases in a simulated rumen environment. The supplements were tested at low and high doses and were evaluated after two incubation times, 5 and 24 h, to verify their effectiveness in modulating the fermentation of maize meal by ruminal microorganisms through the measurement of pH, VFA, ammonia N, DMD and STD. The fixed effect incubation time (5 *vs* 24 h) was found to be highly significant (P<0.001) for all of the investigated variables: longer incubation time led to a greater fermentation of the substrate and to the accumulation of VFA in the tubes, reducing the ruminal fluid pH. After 5 h of incubation, the supplements were found to significantly affect the pH, total VFA, the ammonia N concentration, the proportions of acetate and propionate and DMD. The effect of dose significantly affected the pH, total VFA concentration and STD (Tables 2 to 8). The Tuckey HSD test, however, did not discriminate between LS*means* of the two doses with respect to each supplement. The interaction supplement×dose (S×D) was never significant. At the dose of 150 mg, supplementation with ACB resulted in the highest pH levels, whereas the highest pH reduction was obtained with BBE and WMB (Table 2). Total VFA concentration, at both doses, was the lowest for ACB compared to the other supplements (Table 3). At the dose of 150 mg ACB led to the highest proportion of acetate and the lowest proportion of propionate, whereas BBE showed an opposite trend (Tables 5 and 6). Butyrate proportion was never affected by supplement or dose (data not reported for brevity). Acarbose, compared to other supplements, resulted in the lowest DMD value at both doses, whilst at 150 mg the highest disappearance was found with WMB. The STD value (Table 8) was not reliable for ACB (as will be shown later) and for other supplements no differences were detected. After 24 h of incubation, the supplement significantly affected the pH, total VFA, acetate proportion and showed a tendency to significance for ammonia N concentration and DMD. The dose led to significant changes in pH, total VFA concentration and STD, even though the Tuckey HSD test did not discriminate between LS*means* of the two doses with respect to each supplement, with the exception of pH and STD (Tables 2 and 8). The pH and STD were also affected by the interaction S×D. Similarly to what found after 5 h of incubation, pH was the highest for ACB at both doses. At 150 mg BBE led to the lowest pH (Table 2). The VFA concentration was minimised by ACB at both doses (Table 3). The proportion of acetate was effectively increased by ACB, whereas it was reduced at its lowest values by BBE and PHA at a dose of 15 mg, and by BBE and CFL at a dose of 150 mg (Table 5). Starch disappearance at the dose of 150 mg, resulted the lowest for BBE followed by CFL and WMB, and finally by PHA that led to the highest value. As previously reported by other authors (McLaughlin et al., 2009a, 2009b; Speight and Harmon, 2010), ACB successfully prevented the production of excessive amounts of VFA, which are primarily responsible for the onset of subacute ruminal acidosis in cattle (De Nardi et al., 2013; Marchesini et al., 2013) and consequently countered the drop of ruminal fluid pH. This effect was likely due to the inhibitory activity of ACB towards α-glycosidase and α-amylase

Table 2. Effect of dosing 5 types of supplement on the *in vitro* rumen pH at 5 and 24 h of incubation time in ruminal fluid.

	Rumen pH			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	6.23^{a,A}	5.83^{a,B}	5.54^{a,B}	5.16^{b,C}
PHA	6.23^{a,A}	6.09^{a,AB}	5.57^{a,B}	5.44^{a,B}
CFL	6.11^{a,A}	6.07^{a,AB}	5.50^{a,B}	5.53^{a,B}
WMB	6.09^{a,A}	5.95^{a,B}	5.49^{a,B}	5.33^{a,BC}
ACB	6.52^{a,A}	6.52^{a,A}	6.40^{a,A}	6.27^{a,A}
SEM	0.071		0.040	
P				
Supplement	**		***	
Dose	*		**	
S x D	n.s.		*	

S, supplement; BBE, bilberry extract from *Vaccinium myrtillus*; PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; D, dose. ^{a,b}Within incubation time, different lowercase superscripts in the same row denote significant differences (P<0.05) with a dose of 15 or 150 mg. $^{A-C}$Within incubation time, different uppercase superscripts in the same column denote significant differences (P<0.05) among five supplements. Control (0 mg of supplement) showed a pH average value (±SD) of 6.23(±0.17) and 5.61(±0.20) at 5 and 24 h, respectively. *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Table 3. Effect of dosing 5 types of supplement on the *in vitro* rumen volatile fatty acids production at 5 and 24 h of incubation time in ruminal fluid.

	VFA, mmol/L			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	4.70^{a,A}	5.47$^{a, A}$	7.10^{a,A}	7.90^{a,A}
PHA	4.79^{a,A}	5.20^{a,A}	6.94^{a,A}	7.50^{a,A}
CFL	5.21^{a,A}	5.43^{a,A}	7.67^{a,A}	8.14^{a,A}
WMB	5.25^{a,A}	5.61^{a,A}	7.80^{a,A}	8.01^{a,A}
ACB	3.70^{a,B}	3.79^{a,B}	4.93^{a,B}	5.31^{a,B}
SEM	0.129		0.167	
P				
Supplement	**		**	
Dose	*		*	
S x D	n.s.		n.s.	

VFA, volatile fatty acids; S, supplement; BBE, bilberry extract from *Vaccinium myrtillus;* PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; D, dose. ^{A,B}Within incubation time, different uppercase superscripts in the same column denote significant differences (P<0.05) among five supplements. Control (0 mg of supplement) showed a pH average value (±SD) of 4.69(±0.85) and 7.01(±1.03) at 5 and 24 h, respectively. *P<0.05; **P<0.01; ns, not significant.

produced by ruminal microorganisms (McLaughlin *et al.*, 2009a, 2009b; Speight and Harmon, 2010). Such activity contributed in the reduction of the fermentation of maize starch and sugars compared to other supplements and Control after both 5 and 24 h of incubation. After 5 h the proportion of propionate, usually high after fermentation of concentrates (McDonald *et al.*, 2011), was the lowest among tested supplements and was paired by the highest proportion of acetate and by the lowest DM disappearance of maize, which confirmed the reduction of its fermentation by ruminal microorganisms. After 24 h, although the pH reduction and the VFA production with ACB remained the lowest, the differences in the proportion of propionate were less evident, likely because of a shift in the microbial community and its fermentation pattern. Results of starch disappearance for ACB were not reliable, in fact the test used for starch determination (#996.11; AOAC, 2000) detected very low glucose level after enzymatic residual starch digestion, due to an interference of the inhibitory activity of residual acarbose toward the enzyme used in the test. This explanation fits with results obtained from fermentation and DM disappearance patterns, according to which STD value of ACB should be low (high residual starch level). The other supplements, compared to ACB, apparently failed to inhibit the VFA production and pH reduction caused by fermentation of the substrate; moreover BBE and WMB seemed to favour maize fermentation rather than inhibit it. The enhancement of carbohydrate fermentation due to BBE and WMB could be at least partially explained by the presence of high amounts of NFC, represented by readily fermentable sugars in these supplements, particularly at the highest dose (Table 1). The fact that, after 5 h starch disappearance did not show any significant differences between supplements at the dose of 150 mg (ACB excluded), confirms that the differences in pH and DMD between supplements are mostly due to the fermentation of readily fermentable sugars belonging to WMB and BBE. Only BBE, after 24 h, at the dose of 150 mg, significantly reduced the STD, showing an inhibitory activity towards starch degradation, thus confirming the inhibition of α-amylase and α-glucosydase found by Johnson *et al.* (2011) for another *Vaccinium* species in an *in vitro* trial. Nevertheless, this effect was concealed by the presence of sugars, which caused pH reduction. In view of a use of this compound as an inhibitor of carbohydrases, sugars should be previously removed. The lack of inhibitory effects of PHA, CFL and WMB on carbohydrases could be due to the interference of a vast array of plant-degrading systems present in the rumen environment, as suggested by Speight and Harmon (2010) and/or to the insensitivity of the microbial amylases to the inhibitory compounds (Kluh *et al.*, 2005). As reported by Selinger *et al*. (1996), there are many diverse enzymatic processes performed by plant cell wall polymer-degrading enzymes, amylases, proteases, phytases and specific plant toxin-degrading enzymes in the rumen. It is also possible that plant-derived α-glycosidase and α-amylase inhibitory compounds with demonstrated efficacy in enzymatic trials (Mosca *et al.*, 2008; Sudha *et al*., 2011) could be functionally altered as a consequence of degradation in the rumen environment. Moreover, Kluh *et al.* (2005) reported that the inhibitory activity of PHA towards amylases of different origins was affected by the molecular structure of the amylase itself and by the environmental pH, which, for mammalian amylases, was found to be optimal at

Table 4. Effect of dosing 5 types of supplement on the *in vitro* ammonia at 5 and 24 h of incubation time in ruminal fluid.

	N-NH4, mg/L			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	$2^{a,A}$	$2^{a,A}$	$107^{a,A}$	$28^{a,A}$
PHA	$3^{a,A}$	$9^{a,A}$	$121^{a,A}$	$159^{a,A}$
CFL	$73^{a,A}$	$25^{a,A}$	$234^{a,A}$	$273^{a,A}$
WMB	$23^{a,A}$	$5^{a,A}$	$210^{a,A}$	$203^{a,A}$
ACB	$56^{a,A}$	$72^{a,A}$	$345^{a,A}$	$392^{a,A}$
SEM	16.7		43.7	
P				
Supplement	*		†	
Dose	n.s.		n.s.	
S x D	n.s.		n.s.	

N-NH4, ammonia; S, supplement; BBE, bilberry extract from *Vaccinium myrtillus*; PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; D, dose. Control (0 mg of supplement) showed a pH average value (±SD) of 18(±28) and 168(±105) at 5 and 24 h, respectively. Data are presented as raw least squares means, and the associated P values are given by statistical analysis of the log-transformed data. †P<0.1; *P<0.05; ns, not significant.

Table 5. Effect of dosing 5 types of supplement on the *in vitro* acetate proportion at 5 and 24 h of incubation time in ruminal fluid.

	Acetate proportion, mmol/L			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	$51.2^{a,A}$	$49.0^{a,B}$	$49.1^{a,B}$	$47.6^{a,B}$
PHA	$51.5^{a,A}$	$51.9^{a,AB}$	$49.1^{a,B}$	$50.0^{a,AB}$
CFL	$53.2^{a,A}$	$50.9^{a,AB}$	$50.0^{a,AB}$	$47.7^{a,B}$
WMB	$52.2^{a,A}$	$52.7^{a,AB}$	$50.1^{a,AB}$	$50.0^{a,AB}$
ACB	$54.1^{a,A}$	$53.9^{a,A}$	$54.3^{a,A}$	$52.8^{a,A}$
SEM	0.62		0.76	
P				
Supplement	*		*	
Dose	n.s.		n.s.	
S x D	n.s.		n.s.	

S, supplement; BBE, bilberry extract from *Vaccinium myrtillus*; PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; D, dose. A,BWithin incubation time, different uppercase superscripts in the same column denote significant differences (P<0.05) among five supplements. Control (0 mg of supplement) showed a pH average value (±SD) of 52.1(±2.9) and 49.4(±3.2) at 5 and 24 h, respectively. *P<0.05; ns, not significant.

approximately 4.5 and unfavourable at 6.9. Similar physiological mechanisms could be involved in the regulation of the inhibitory activity of the tested supplements towards the bacterial enzymes in the rumen.

In the rumen, up to 70% of the dietary protein is degraded by the combined action of microbial proteases and peptidases. Released amino acids (AA) are used for the synthesis of microbial proteins or metabolised to generate ammonia for *de novo* synthesis of AA (Selinger *et al.*, 1996). In this study, the maize protein seems to be degraded to ammonia N to the greatest extent following treatment with ACB, especially at the highest dose. Possible shifts in microbial community may explain this result. The partial inhibition of α- glycosidase and α-amylase and consequently, the lack of an available energy-rich substrate, could have reduced the multiplication of starch- and sugar-fermenting microorganisms and favoured ammonia–producing microorganisms that utilise protein, peptides and AA as energy and nitrogen sources (Krause śand Russell, 1996; Szumacher-Strabel and Cie lak, 2010). An opposite trend was observed following BBE treatment, especially after 24 h of incubation, which led to low ammonia concentrations. This result could be explained by the rapid fermentation of sugars and starch, which could provide energy for microbial protein synthesis using ammonia and AA as nitrogen sources (McDonald *et al.*, 2011). With regard to the dose effect, the highest concentration of supplements led to an increment of VFA level and to a higher reduction of pH, suggesting that the supplements added were at least partially fermented by ruminal microorganisms, leading to an increase in fermentation products. With respect to the time of action of the tested compounds and their possible use in live animals, only ACB seems to be fast enough to inhibit carbohydrase activity within 5 h from the meal, preventing the drop of ruminal pH and its possible detrimental effects on cellulolytic bacteria and ruminal epithelium (Marchesini *et al.*, 2013). The BBE supplement, instead, resulted effective in inhibiting STD only after a period of 24 h.

Conclusions

Among the supplements tested, only ACB effectively prevented the drop in pH and limited DM disappearance and the production of VFA through the inhibition of maize meal fermentation, whereas among plant-derived supplements, BBE showed an activity against starch degradation, although this effect was concealed by the fermentation of sugars present in the supplement. The remaining compounds, in line with the Control, failed to inhibit bacterial carbohydrases during in batch rumen fermentation. The differences found in ammonia N concentration and VFA proportion among supplements suggest that some of these compounds could affect the microbial composition at the ruminal level and consequently the fermentation pattern. Although the effects of amylase/glucosidase inhibitors of different origins on mammalian enzymes have been studied extensively, their effects on the fermentation pattern of complex microbial communities, such as that of the rumen, require further investigation. An improved understanding of the mechanisms involved in the manipulation of ruminal fermentation could improve animal performance and reduce the risk of metabolic diseases such as ruminal acidosis.

Table 6. Effect of dosing 5 types of supplement on the *in vitro* propionate proportion at 5 and 24 h of incubation time in ruminal fluid.

	Propionate proportion, mmol/L			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	29.4^{a,A}	32.7^{a,A}	29.1^{a,A}	33.4^{a,A}
PHA	29.3^{a,A}	29.8^{a,AB}	29.1^{a,A}	29.5^{a,A}
CFL	28.0^{a,A}	29.7^{a,AB}	29.9^{a,A}	29.7^{a,A}
WMB	29.8^{a,A}	29.4^{a,AB}	28.9^{a,A}	30.0^{a,A}
ACB	27.4^{a,A}	26.9^{a,B}	26.6^{a,A}	28.6^{a,A}
SEM	0.70		1.04	
P				
Supplement	*		n.s.	
Dose	n.s.		n.s.	
S x D	n.s.		n.s.	

S, supplement; BBE, bilberry extract from *Vaccinium myrtillus*; PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; D, dose. A,BWithin incubation time, different uppercase superscripts in the same column denote significant differences (P<0.05) among five supplements. Control (0 mg of supplement) showed a pH average value (±SD) of 29.8(±6.1) and 29.8(±6.4) at 5 and 24 h, respectively. *P<0.05; ns, not significant.

Table 7. Effect of dosing 5 types of supplement on the *in vitro* dry matter disappearance at 5 and 24 h of incubation time in ruminal fluid.

	DMD			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	0.35^{a,A}	0.35^{a,AB}	0.68^{a,A}	0.71^{a,A}
PHA	0.33^{a,A}	0.33^{a,AB}	0.65^{a,A}	0.64^{a,A}
CFL	0.33^{a,A}	0.33^{a,AB}	0.66^{a,A}	0.66^{a,A}
WMB	0.33^{b,A}	0.38^{a,A}	0.67^{a,A}	0.69^{a,A}
ACB	0.26^{a,B}	0.28^{a,B}	0.57^{a,A}	0.55^{a,A}
SEM	0.013		0.046	
P				
Supplement	**		†	
Dose	n.s.		n.s.	
S x D	n.s.		n.s.	

DMD, dry matter disappearance; S, supplement; BBE, bilberry extract from *Vaccinium myrtillus*; PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; D, dose. a,bWithin incubation time, different lowercase superscripts in the same row denote significant differences (P<0.05) with a dose of 15 or 150 mg. A,BWithin incubation time, different uppercase superscripts in the same column denote significant differences (P<0.05) among five supplements. Control (0 mg of supplement) showed an average value (±SD) of 0.34(±0.07) and 0.66(±0.12) at 5 and 24 h, respectively. **P<0.01; †P<0.1; ns, not significant.

Table 8. Effect of dosing 5 types of supplement on the *in vitro* starch disappearance at 5 and 24 h of incubation time in ruminal fluid.

	STD			
	5 hours		24 hours	
	15 mg	150 mg	15 mg	150 mg
BBE	$0.43^{a,A}$	$0.26^{a,A}$	$0.76^{a,A}$	$0.47^{b,B}$
PHA	$0.41^{a,A}$	$0.29^{a,A}$	$0.76^{a,A}$	$0.70^{a,A}$
CFL	$0.26^{a,A}$	$0.33^{a,A}$	$0.63^{a,A}$	$0.67^{a,AB}$
WMB	$0.28^{a,A}$	$0.12^{a,A}$	$0.72^{a,A}$	$0.65^{a,AB}$
ACB	n.d.	n.d.	n.d.	n.d.
SEM	0.047		0.036	
P				
Supplement	n.s.		n.s.	
Dose	*		*	
S x D	n.s.		*	

STD, starch disappearance; S, supplement; BBE, bilberry extract from *Vaccinium myrtillus;* PHA, phaseolamin from *Phaseolus vulgaris*; CFL, seed extract of common flax from *Linum usitatissimum*; WMB, extract of the white mulberry (*Morus alba*) leaf; ACB, acarbose; nd, not determined; D, dose. a,bWithin incubation time, different lowercase superscripts in the same row denote significant differences (P<0.05) with a dose of 15 or 150 mg. A,BWithin incubation time, different uppercase superscripts in the same column denote significant differences (P<0.05) among five supplements. Control (0 mg of supplement) showed an average value (±SD) of 0.39(±0.11) and 0.73(±0.07) at 5 and 24 h, respectively. *P<0.05; ns, not significant.

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CHAPTER 7

Use of dicarboxylic acids and polyphenols to attenuate reticular pH drop and acute phase response in dairy heifers fed a high grain diet

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Use of dicarboxylic acids and polyphenols to attenuate reticular pH drop and acute phase response in dairy heifers fed a high grain diet

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Abstract

Background: The aim of this study was to determine the ability of two feed additives, a fumarate-malate (FM) and a polyphenol-essential oil mixture (PM), in attenuating the drop of ruminal pH and the metabolic and immune response resulting from an excessively high grain diet. Six heifers were used in a 3 × 3 Latin square experiment and fed a low starch (LS) diet for 14 d, followed by a high starch (HS) diet for 8 d (NDF 33.6%, starch 30.0% DM). In the last 5 days of each period, barley meal was added to decrease rumen pH. During HS feeding all animals were randomly assigned to one of the following three dietary treatments: no supplement/control (CT), a daily dose of 60 g/d of FM, or 100 g/d of PM. Reticular pH was continuously recorded using wireless boluses. On d 21 of each period, rumen fluid was collected by rumenocentesis (1400 h), together with blood (0800 h) and fecal samples (0800, 1400, and 2100 h).

Results: The correlation coefficient of pH values obtained using the boluses and rumenocentesis was 0.83. Compared with CT and PM, the FM treatment led to a lower DMI. Nadir pH was lowest during CT (5.40, 5.69, and 5.62 for CT, FM and PM, respectively), confirming the effectiveness of both supplements in reducing the pH drop caused by high grain feeding. This result was confirmed by the highest average time spent daily below 5.6 pH (199, 16 and 18 min/d) and by the highest acetate to propionate ratio of the CT fed heifers. The PM decreased the concentrations of neutrophils (2.9, 3.2, and 2.8 10^9/L) and acute phase proteins: SAA (37.1, 28.6 and 20.1 μg/mL), LBP (4.1, 3.8, and 2.9 μg/mL), and Hp (675, 695 and 601 μg/mL). Free lipopolysaccharides (LPS) were detected in blood and feces, but their concentrations were not affected by treatments, as the remaining blood variables.

Conclusions: Data suggest that both additives could be useful in attenuating the effects of excessive grain feeding on rumen pH, but the PM supplement was more effective than FM in reducing the inflammatory response compared to CT.

Keywords: Reticular pH, Acute phase protein, Fumarate-malate, Polyphenol, High grain diet, Heifer

Background

In the grain-based and energy-dense diets typically fed to highly productive dairy cows, the rapid ruminal fermentation of starch and sugars can lead to an accumulation of volatile fatty acids (VFA) and/or lactate, which causes a drop of pH and a shift in the balance of rumen microorganisms [1,2]. Especially, when switching from a low-energy high-forage diet to a high-energy early lactation diet, the rate of microbial VFA production exceeds the rate at which they can be cleared from the rumen [3,4]. As a result, VFA accumulates and the rumen pH declines [5]. The condition in which pH is depressed for prolonged periods each day is defined as subacute ruminal acidosis (SARA). This is an ongoing and costly digestive disorder of dairy cows [6,7], affecting rumen fermentation, production and immune response [8-10]. Good management and nutritional strategies alone [8,5] are not always enough to avoid the onset of SARA in high yielding dairy cows and for this reason other preventative measures have been suggested. This

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included physical manipulation of fiber and grain particle size [8,11,12], the inclusion of antibiotics in the diet [13], the use of yeasts and probiotic bacteria [14,15], and the addition of dicarboxylic acids [16], flavonoids [17] or essential oils [18] to manipulate rumen microbial communities and subsequently ruminal fermentation.

The dicarboxylic acids malate and fumarate have been proposed as modifiers of ruminal fermentation and as an alternative to antibiotics [19,16]. It has been suggested that these acids may increase the activity of the succinatepropionate metabolic pathway in several rumen bacteria which results in increased lactic acid uptake and production of propionate [2,20]. Flavonoids, essential oils and other plant-derived compounds have been proposed as feed supplements for their anti-inflammatory, antioxidant, and antimicrobial properties [21,18,22]. In addition, flavonoids have been reported to be effective in preventing pH reduction through modifying the activity of lactating-consuming bacteria and promoting the growth of propionate-producing bacteria [17]. Moreover, essential oils are known to have antimicrobial properties, and have been suggested to act as rumen fermentation modifiers [18].

The aim of this study was to determine the effects of two feed additives, a fumarate-malate mixture and a polyphenol-essential oil mixture, in attenuating the drop of rumen pH and the changes in metabolites and inflammatory markers in blood and rumen fluid due to feeding high grain diets to heifers.

Methods

Animals and experimental design

The experimental protocol and all the procedures used in this study were approved by the Animal Ethics Committee of the University of Padova, Italy (CEASA, approval number 73/2012).

Six purebred Italian Holstein-Friesian non-pregnant heifers with an average body weight (BW) of 556 ± 33 kg (mean ± SD) were used in a 3 × 3 Latin square design. Heifers were born in a dairy farm located in the lowland of Vicenza (Veneto Region, Italy) where the experimental trial took place. After the experiment, heifers were inseminated to begin their normal productive life as dairy cows. Animals were randomly assigned to one of the three dietary treatments during each period. Each period lasted 22 d: 14 d of an adjustment phase followed by 8 d of data collection. Heifers were kept in individual pens in loose housing conditions and had unlimited access to fresh water. All of the heifers were examined by a veterinarian to evaluate their health status throughout the trial.

Dietary treatments

During each period heifers were fed a low starch (LS) diet for 14 d ad libitum, followed by a high starch (HS) diet for 8 d: from d 15 to d 22 (Table 1). Diets were provided as TMR once daily at 0800 h. During the HS feeding heifers were offered one of three dietary treatments: i) no supplement, CT treatment; ii) a daily dose of 60 g of fumarate-malate mixture (RumenStabiliser®, DR. Eckel, Niederzissen, Germany), FM treatment; iii) a daily dose of 100 g of polyphenol-essential oil mixture (Anta®Phyt RU, DR. Eckel, Niederzissen, Germany), PM treatment.

The FM supplement is an organic acid and buffer blend made of magnesium fumarate, malic acid, sodium acetate and sodium bicarbonate, whereas PM is a blend made of natural plant extracts, characterised by a high content of phenolic compounds comprising mostly flavonoids (1.88 mg/g). The amount of dicarboxylic acids and flavonoids reported to be effective in the modification of ruminal fermentations in the literature [23,24,17] was taken into account in determining the supplement doses. The two supplements were given together with approximately 1 kg of TMR and their complete intake

Table 1 Ingredients and proximate composition of diets fed to heifers

Item	Diet[1]	
	LS	HS
Ingredients, % of DM		
Corn meal (0.5 mm)	23.0	34.0
Hay	24.0	22.0
Dehydrated alfalfa hay	18.0	16.0
Extruded soybean hull	7.0	2.0
Barley meal	7.0	9.0
Straw	6.5	5.5
Molasses	2.0	2.0
Sugar beet dry pulps	4.0	1.0
Corn gluten meal	6.0	2.0
Sunflower	-	2.0
Soybean meal	-	2.0
Vitamin and mineral mix	1.5	1.5
Extruded de-hulled soybean	1.0	1.0
Proximate composition		
DM, %	88.9	88.8
Crude protein, % of DM	12.5	12.5
Crude fat, % of DM	3.5	3.3
NDF, % of DM	39.8	33.6
Crude ash, % of DM	7.8	7.4
ADF, % of DM	21.4	19.2
NFC, [2]% of DM	36.4	43.2
Starch, % of DM	24.0	30.0

Diets: LS = low starch; HS = high starch.
NFC = 100 – (NDF + crude protein + crude fat + crude ash).

was verified by an operator before distributing the rest of the ration.

With the aim to induce a drop in rumen pH below 5.6 for more than 3 h/d, the threshold pH established for SARA [6], from the d 18 to the d 22, barley meal (32.5% NDF and 53.1% starch on DM) was top dressed on the TMR. The quantity of barley meal was gradually increased from 0.5 to 1.5 kg (with 250 g increment per day) to prevent from a fast drop of pH that could have led to acute ruminal acidosis. The barley meal provision and the control of its intake were performed using the same technique described for the supplements in order to guarantee the same level of intake throughout the treatments.

Feed intake and feed analyses

The weight of the feed offered and refused was recorded daily during the HS feeding. Samples of both the LS and HS diets were collected twice for each period and analyzed for proximate composition (Table 1). Feed samples were dried at 60°C for 48 h and ground to pass through a 1-mm screen and then analysed for DM, crude protein (CP), crude fat and crude ash according to AOAC [25], whilst NDF and ADF were analyzed according to Van Soest et al. [26] using α-amylase and a Fibre Analyser (ANKOM/ 2000; ANKOM Technology, New York, NY, USA). The starch content was determined using high performance liquid chromatography equipped with a LC 9A pump, SIL auto sampler and the RID-10 A model (Shimadzu, Tokyo, Japan); separations were achieved using a 300 × 7.8 mm Aminex HPX-87H column and one pre-column (Micro Guard Cation H 30 × 4.6 mm, Bio-Rad, Hercules, CA, USA) at 40°C [27].

Sampling and analysis of ruminal fluid and reticular pH

The pH of the reticulum was continuously measured during the entire trial using wireless boluses (SX-1042, SmaXtec Animal Care GmbH, Graz, Austria). The boluses were calibrated and delivered orally in the reticulum using a balling gun and their positions were verified using ultrasound measurement. The pH readings were recorded every 10 min [28].

The pH data of the last two days of each period (d 21 and 22), measured by the bolus in each animal, were summarized daily as the average, maximum and nadir pH. With the purpose to make comparison with data present in literature, the amounts of time per day that the pH was below three ruminal pH thresholds (pH <5.6, pH <5.8 and pH <6.3) were determined for each heifer during the three experimental periods, as described by Gozho et al. [29].

In this study, the pH threshold values were selected because the duration of the rumen pH below 5.6 is related to an increase of the intensity of the inflammatory acute phase response [30]; pH <5.8 is harmful to ruminal cellulolytic bacteria [31]; pH <6.3 is proposed by Sato et al. [32] for SARA determination from reticular fluid, given that the reticular pH is higher than the ruminal pH, due to mixing and dilution with saliva [33].

Rumenocentesis was performed at 1400 h (6 hours after TMR distribution) on d 21 of each period using a 13G 105-mm needle [34]. The pH of ruminal fluid was measured immediately using a portable pH meter (Piccolo, Hanna Instruments, Villafranca Padovana, Italy) and compared to the average pH values recorded by the boluses just prior to and immediately after the rumenocentesis time [7]. Rumen fluid samples were strained through 4 layers of sterile cheesecloth and were collected and divided into 2 portions. The first portion of each sample was transferred into a 50-mL sterile tube and kept on ice until the processing required for lipopolysaccharide (LPS) determination, as described by Li et al. [9]. For LPS, rumen fluid samples were centrifuged at 12,000 × g for 40 min at 4°C; the supernatant was aspirated, filtered using 0.22-μm sterile, pyrogen free filter (Millex, Millipore Corporation, Bedford, MA, USA) and collected into depyrogenated glass tubes (heated at 200°C for 2.5 h). The samples were heated at 100°C for 30 min, cooled at room temperature for 10 min and stored at −20°C until analysis. Free LPS in rumen fluid was measured by a chromogenic kinetic Limulus amebocyte lysate (LAL) assay (50−650 U, Kinetic-QCL, Lonza Group Ltd., Basel, Switzerland) in a 96-well microplate using an incubating microplate spectrophotometer (Synergy H4 Hybrid Multi-Mode Microplate Reader, Bio-Tek, Instruments, Inc., Winooski, VT, USA). Rumen fluid samples were diluted 1:67,100 using LAL water pyrogen-free (LAL Reagent Water, Lonza Group Ltd., Basel, Switzerland), with the final dilution being made of 50% diluted sample and 50% β-glucan blocker (N.190, Lonza, Walkersville, MD, USA).

The second portion of each rumen fluid sample (5 mL) was centrifuged at 3,000 × g for 15 min at 4 °C, for VFA, lactate and ammonia N analyses. Samples were acidified with 0.6 M HCl to inhibit microbial activity and minimize volatilization (dilution 5:1) and stored at −20°C until analysis.

After thawing, samples were centrifuged at 4,000 × g for 30 min at 4°C and the supernatants were filtered using 0.45-μm Phenex-RC filters (Phenomenex, Castel Maggiore, Italy). One subsample of the filtrate was analysed for ammonia N using a SmartChem 200 spectrophotometer (Unity Scientific, Brookfield, CT, USA). For the VFA and lactate analysis, a second subsample was injected into an HPLC system complete with an LC 9 A Shimadzu pump, a SIL 10A auto sampler and a RIDmodel Shimadzu 10A detector (Shimadzu, Tokyo, Japan). Analytes separation was performed at 40°C using an Aminex HPX-87H column (300 × 7.8 mm) and one pre

column (Bio-Rad, Hercules, CA, USA). Class VP software was used for data collection and integration. For the complete HPLC analysis, a 30 min isocratic program was run with 0.025 N H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Peaks of analytes were identified by comparing the retention times of standard mixtures to those of the samples and quantification was based on peak area measurements that were compared with that of an external standard.

Blood collection and analysis

Blood samples (20 mL) from the jugular vein were collected from each animal before the feed delivery at 0800 h on d 21 of each period into lithium-heparin, K3 EDTA and tubes without anticoagulant (Vacuette, Greiner Bio-One, Kremsmuenster, Austria). The blood from the K3 EDTA tubes and one subsample of lithium-heparin preserved blood were refrigerated (4°C) and analyzed within 1 h for a complete blood cell count and blood gas analysis, respectively. The other subsamples were immediately centrifuged at 1,500 × g for 15 min at 4°C for plasma and serum separation, and were preserved at −80°C until analysis.

Complete blood cell count with leukocyte formula was performed using an automated cell counter (Cell Dyn 3500, Abbott Laboratories, Abbott Park, IL, USA). Blood gas analysis was performed within 1 h from the collection in a calibrated blood gas analyzer (Synthesis 15, IL Instrumentation Laboratory SpA, Milano, Italy) and blood pH, partial pressure of carbon dioxide, partial pressure of oxygen, and the percentage of oxyhemoglobin and reduced hemoglobin were determined. The bicarbonate levels and measured oxygen saturation were calculated. Measurements were performed as recommended by the National Committee of Blood Laboratory Standards [35]. The plasma was analyzed for the hematological profile: glucose, cholesterol (CHOL), non-esterified fatty acids (NEFA), β-hydroxybutyrate (β-HB), aspartate aminotransferase (AST), γ-glutamyl transferase (γGT) by using a Roche Cobas C501 automatic analyzer (Roche Diagnostics, Indianapolis, IN, USA). Plasma was also analyzed for LPS, interleukin 6 (IL-6) cytokine and acute phase proteins: serum amyloid A (SAA), LPS binding protein (LBP) and haptoglobin (Hp). The SAA, LBP and Hp were measured using the following commercially available ELISA kits, respectively, as described by Gozho et al. [36] and Khafipour et al. [37]: TP802-2 (Tridelta Diagnostics Ltd., Maynooth, Co. Kildare, Ireland), HK503 (Hycult Biotech Inc., Plymouth Meeting, PA, USA), TP801-Mk2 (Tridelta Diagnostics Ltd., Maynooth, Co. Kildare, Ireland) and ESS0029 (Thermo Scientific, Rockford, IL, USA). A chromogenic LAL assay (Kinetic-QCL™, Lonza Group Ltd., Basel, Switzerland) was used to measure the concentration of LPS in plasma as described by Khafipour et al. [37] and Li et al. [9]. Samples were diluted 1:4 with LAL Reagent Water. Samples were incubated at 70°C for 30 min. Heated samples (100 µL) were added to a 1:7 diluted 10 mM $MgCl_2$ solution (Lonza Group Ltd., Basel, Switzerland) and Pyrosperse® (N188, Lonza Group Ltd., Basel, Switzerland).

Fecal sampling and analyses

Fecal samples were collected from the rectum at 0800 , 1400 and 2100 h on the d 21 of each period. The pH was measured immediately using a portable pH meter (Piccolo, Hanna Instruments, Villafranca Padovana, Italy). About 40 g sample was processed for LPS analyses using the same procedure adopted for rumen fluid, but with a dilution of 1:26,000 [38]. These samples were stored at −20°C until analysis.

Statistical analysis

The DMI and reticular pH data were analyzed using the MIXED procedure with a compound symmetry structure. The linear model considered the fixed effect of dietary treatment (CT, FM and PM), period, day (repeated measure) and their interactions. Heifer was included as random effect. Ruminal and blood data were analyzed according to the same model but without the day effect (data recorded on d 21). Moreover, feces data were also analyzed according to the described model, but the day was replaced by daily sampling time effect (0800, 1400 and 2100 h). If significant treatment effects were detected ($P < 0.05$), the LSmeans were compared using the probability of differences (PDIFF) option and the Tukey adjustment test. To obtain a normal distribution and homogeneous residual error, ruminal LPS, blood LPS and IL-6 data were log transformed. Means and confidence intervals were then reported in tables after antilog transformation.

The average amount of time for each heifer with a reticular pH below the three established pH thresholds (<5.6, <5.8 and <6.3) were not normally distributed even after transformation. Thus, these data were tested using the non-parametric Kruskal-Wallis criteria using the Dunn′s multiple pairwise comparisons. Pearson′s correlation coefficient (PROC CORR) was assessed between the reticular and ruminal pH measurements.

All of the statistical analyses were performed using SAS software (2010, release 9.3; SAS Institute Inc., Cary, NC, USA). The effects were considered significant at $P < 0.05$ and trends were discussed at $0.05 < P < 0.10$.

Results

Weight gain and DMI

At the end of the trial, the heifers weighed an average of 625 ± 37 kg and the average daily gain was 1.04 ± 0.09 kg/d. The DMI was affected by the treatment ($P = 0.021$) and was the lowest on the FM diet (Table 2).

Table 2 Effect of dietary treatment on DMI, reticular pH, daily average of time spent below the three reticular pH thresholds, ruminal volatile fatty acid (VFA) N-NH3 and lipopolysaccharides (LPS)

	Treatment[1]			SEM	P-value
Item	CT	FM	PM		
DMI, [2] kg/d	14.5[a]	13.4[b]	14.7[a]	0.62	0.021
Reticular pH					
Average	6.04	6.11	6.09	0.067	0.466
Maximum	6.61	6.54	6.55	0.063	0.569
Nadir	5.40[b]	5.69[a]	5.62[ab]	0.106	0.037
pH <5.6, [3] min	199[a]	16[b]	18[b]	-	0.022
pH <5.8, [3] min	360	190	171	-	0.311
pH <6.3, min	1156	1118	1071	86.4	0.546
VFA, mM					
Acetate	59.2	60.9	60.6	3.60	0.911
Propionate	26.5	30.8	27.9	2.07	0.249
Butyrate	10.5	10.7	9.8	0.69	0.683
Ac:Pr[4]	2.05[α]	1.66[β]	1.77[αβ]	0.125	0.048
$N\text{-}NH_3$, mg/dL	50.1	54.5	31.1	9.70	0.303
LPS[5, 6], $\times 10^3$ EU/mL	15.7	6.0	8.9	(5.3–16.7)	0.172

[1]Treatments: control diet (CT); fumarate-malate mixture (FM); polyphenol-essential oil mixture (PM).
[2]For DMI the repeated effect of day was significant (P <0.001).
[3]P-values and superscript letters ([a, b]: P <0.05) are given by using the non-parametric Kruskal–Wallis test and the Dunn's multiple pairwise comparison.
[4]Acetate to propionate ratio.
[5] Statistical analysis was conducted on natural logarithm (ln) transformed data that are presented as ln back transformed and 95%-confidence interval in brackets.
[6]LPS were reported as endotoxin unit.
[a, b]Means with different superscripts within a row differ (P <0.05). [α, β] Means with different superscripts within a row differ (P <0.10).

Moreover the DMI was affected by day and was found to increase from the 18th (14.2 kg/d) to the 21st d (15.1 kg/d) and then slightly decrease on the 22nd d (14.7 kg/d).

pH, VFA, $N\text{-}NH_3$ and free LPS in rumen fluid

The correlation coefficient (r) between the pH values obtained using the reticular boluses and rumenocentesis was 0.83 (P <0.001). The dietary treatment significantly affected nadir pH, the acetate to propionate ratio and the time spent below pH 5.6, but not the concentrations of VFA, ammonia N, LPS, mean and maximum pH and the time spent below pH 5.8 and 6.3 (Table 2). The FM diet led to the highest nadir pH (P = 0.037), whereas both FM and PM significantly reduced the time spent below 5.6 compared to CT (199 vs. 16 vs. 18 min/d, for CT, FM and PM respectively; P = 0.022). The acetate to propionate ratio (Table 2) was the highest with CT and the lowest with FM (P = 0.048). The concentration of lactate was almost negligible (<0.01 mM) and was not affected by treatment

Blood variables

Peripheral blood concentrations of acute phase proteins (SAA, LBP and Hp) were affected by treatment, whereas concentrations of IL-6 and LPS, total blood cell count, blood gas and haematological profile variables did not show any differences among treatments. An exception was neutrophils (NEU) that were significantly lower (P = 0.084) in the PM treatment (Tables 3 and 4). The PM treatment decreased the concentration of SAA (P = 0.036), LBP (P = 0.048) and Hp (P = 0.084), whereas FM showed intermediate values between PM and CT for SAA and LBP.

Fecal variables

The pH and LPS concentration of feces were not affected by dietary treatment (Table 3), whereas fecal pH values were affected by time (P =0.042) and were 6.38 , 6.71 and 6.69 at 0800, 1400 and 2100 h, respectively.

Discussion

Our objective was to test if the addition of a supplement based on fumarate-malate (FM) or a polyphenol-essential oil mixture (PM) to an high grain and potentially subacute rumen acidosis-inducing diet alters ruminal fermentations and attenuates the reticular pH drop, the production of LPS and their consequences on the immune response and on biochemical and blood gas profiles that result from feeding this diet. For this purpose heifers were preferred

Table 3 Effects of dietary treatment on concentrations of serum amyloid A (SAA), lipopolysaccharide binding protein (LBP), haptoglobin (Hp), interleukin-6 (IL-6), blood lipopolysaccharides (LPS), and fecal pH and LPS

Item	Treatment[1]			SEM	*P*-value
	CT	FM	PM		
Blood					
SAA, µg/mL	37.1[a]	28.6[ab]	20.1[b]	5.21	0.036
LBP, µg/mL	4.1[a]	3.8[ab]	2.9[b]	0.65	0.048
Hp, µg/mL	675[αβ]	695[α]	601[β]	42.9	0.084
IL-6,[2] ng/mL	0.90	1.23	0.83	(0.0–4.1)	0.260
LPS,[3] EU/mL	0.35	0.36	0.42	0.082	0.387
Feces[4]					
pH	6.60	6.56	6.65	0.057	0.697
LPS,[2,3] x 10^3 EU/mL	10.9	5.4	10.9	(5.4-13.9)	0.168

[1]Treatments: control diet (CT); fumarate-malate mixture (FM); polyphenol-essential oil mixture (PM).
[2]Statistical analysis was conducted on natural logarithm (ln) transformed data that are presented as ln back transformed and 95%-confidence interval in brackets.
[3]LPS were reported as endotoxin unit.
[4]For feces variables, the statistical model included also the repeated effect of daily sampling time (3 levels: 0800 vs. 1400 vs. 2100). The effect was significant for pH (*P* =0.042), but not for LPS (*P* =0.128).
[a,b]Means with different superscripts within a row differ (*P* <0.05).
[α, β]Means with different superscripts within a row differ (*P* <0.10).

Table 4 Effects of dietary treatment on blood pH, count, gas and haematological profile

Item[2]	Treatment[1]			SEM	P-value
	CT	FM	PM		
pH	7.42	7.42	7.41	0.009	0.815
Red blood cells, 10^{12}/L	6.6	6.5	6.6	0.27	0.441
White blood cells, 10^9/L	8.2	8.4	8.1	0.24	0.726
Neutrophils, 10^9/L	$2.9^{\alpha\beta}$	3.2^{α}	2.8^{β}	0.27	0.084
Lymphocytes, 10^9/L	4.1	3.9	3.9	0.19	0.835
Monocytes, 10^9/L	0.82	0.87	0.95	0138	0.148
Basophils, 10^9/L	0.10	0.09	0.10	0.021	0.794
Eosinophils, 10^9/L	0.31	0.35	0.33	0.102	0.908
Hemoglobin, g/dL	11.4	11.2	11.5	0.26	0.128
Hematocrit, %	32.2	31.8	32.4	0.64	0.318
Platelets, K/µL	243	280	241	44.8	0.359
pCO_2, mmHg	48.0	48.9	47.1	1.36	0.538
pO_2, mmHg	60.2	71.1	59.8	5.66	0.299
HCO^-_3, mmol/L	31.2	32.0	30.5	0.62	0.235
Oxyhemoglobin, %	90.5	91.4	89.0	2.13	0.701
Reduced hemoglobin, %	6.8	6.0	8.4	2.12	0.693
sO_2m, %	93.1	94.1	91.4	2.19	0.681
Glucose, mmol/L	4.45	4.55	4.50	0.137	0.818
Cholesterol, mmol/L	3.3	3.4	3.3	0.24	0.957
NEFA, meq/L	0.07	0.57	0.07	0.403	0.637
β-HB, mmol/L	0.33	0.29	0.41	0.049	0.325

[1]Treatments: control diet (CT); fumarate-malate mixture (FM); polyphenol-essential oil mixture (PM).
[2]pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; HCO^-_3, bicarbonate level; sO_{2m}, measured oxygen saturation; NEFA, non-esterified fatty acids; β-HB, β- hydroxybutyrate.
$^{\alpha,\beta}$Means with different superscripts within a row differ (P <0.10).

to dairy cows to avoid the carry-over effects from the feeding on high grain diets in previous lactations. Both the addition of FM and PM reduced the drop of reticular nadir pH and the daily average time spent by the heifers below pH 5.6 when compared to CT diet. The heifers fed the latter diet spent 199 min below pH 5.6, which is more than the threshold for SARA proposed by Gozho et al. [30], even though the pH in the reticulum is reported to be higher than in the rumen [32,33]. Treatment did not affect the fecal pH, which confirms that ruminal pH is not closely related to the latter [8].

The concentration of LPS found in the rumen of CT fed animals (raw data, 38,300 EU/mL) was similar to that reported by other authors after an episode of SARA induced by feeding high grain diets [30,39]. This means that the high grain diet led to a drop of rumen pH sufficient to trigger the production and the accumulation of LPS in rumen fluid. Although in other studies [37,9] high grain diets led to concentrations of ruminal LPS higher than that found in the present trial, it must be remembered that previous studies used cows instead of heifers. Heifers have lower feed intakes and are also subjected to less nutritional and metabolic stresses compared to cows, which may explain the relatively lower rumen LPS concentrations in our study.

The LPS are bacterial endotoxins which, when the mucosa of the digestive tract is damaged, can translocate into the bloodstream [40] and induce systemic immune response and metabolic alterations which can compromise animal health and performance [41].

The FM and PM treatments reduced the time spent below pH 5.6 to the same extent, but this effect, for FM fed heifers, could be partially due to the reduction in DMI that was 7.5% lower than that found for CT and PM diets. This reduction is in agreement with that found by other authors [42,24] after the administration of dicarboxylic acids, although the dietary inclusion rate of the fumarate-malate based supplement in our study was much lower than that reported to cause DMI reduction [24]. The DMI was also affected by day, increasing from d 18 to d 21 and then decreasing on d 22. This effect is probably caused by the preference for barley meal whose amount was raised gradually along the last five days of each period. The reduction of DMI in d 22 corresponded to the drop of reticular pH at this time. According to the literature [17,20,43] the mechanisms of action through which both fumarate-malate and polyphenols should reduce the drop of rumen pH are related to a change in the fermentation pattern and to the increasing of lactate utilization by some anaerobic lactate consuming bacteria.

Martin [20] reported that fumarate and malate are intermediates of the citric acid cycle and that they may provide an electron sink for H_2 that allows for increased lactate utilization by strictly anaerobic bacteria which use the succinate-propionate pathway to synthesize succinate and (or) propionate. Other authors [17] found that flavonoids modify the activity of some lactate consuming bacteria, which rapidly metabolize lactate to VFA, thereby, preventing lactate accumulation in the rumen. However, in this study, the concentration of lactic acid was found to be negligible. This probably means that lactic acid was produced and immediately converted into VFA, as suggested by some authors, thereby preventing any accumulation of lactate in the rumen [43,44].

The action of both supplements in the rumen microbial fermentation pattern is also supported by the reduction in the acetate to propionate ratio by FM and PM compared to CT. In the rumen, the ratio between different products of carbohydrate fermentation depends on the hydrogen concentration, due to microbial interspecies hydrogen transfer [45]. The possible effect of some additives on hydrogen -producing and hydrogen - consuming microorganisms, can lead the pyruvate to be converted to

different VFA, CO_2, hydrogen and intermediary products, according to a fermentation pattern that varies with microbial species [45]. The PM treatment resulted in the greater attenuation of the increase in the concentrations of APP (SAA, LBP and Hp) in blood after high grain feeding. The increase of APP, plasma proteins produced mainly from the liver and used as sensitive markers of the inflammation, is the expression of a systemic and innate reaction of the organism to inflammation triggered by external (pathogens, toxins, etc.) or internal (tissue damage, etc.) stimuli [46]. Many APP, including SAA and Hp, are poorly specific for pathogens and toxins [47]. However, in our study their increase is likely due to the translocation of LPS out of the digestive tract into the portal circulation [36,37,48]. As routinely checks by a veterinarian excluded other possible causes of inflammation, like laminitis or respiratory disorders. The SAA has different functions but mainly modulates innate immune reactions and in particular the migration of monocytes and neutrophils, whereas Hp has antiinflammatory properties; LBP is triggered by bacterial infections and helps in the neutralization of LPS and in the activation of a cascade of reactions that leads to the release of cytokines, among which IL-6, that are necessary for the activation of the immune system [10,46,47]. The FM showed intermediate values among treatments for the concentrations of SAA and LBP, but the highest value for Hp, proving that its effect in attenuating the inflammation process, due to the rumen pH drop, is lower than that of PM. The concentration of LBP was lower than that found by other authors after high grain feeding [39,48]. This could be possibly related to the fact that heifers are not subjected to as many chronic disorders, and nutritional and metabolic stresses, that could may have occurred in cows during previous lactations, and thereby affect the concentrations of LBP [46].

The lack of differences among treatments in LPS and IL-6 blood concentrations suggests that these variables are less sensitive to the systemic effects of rumen pH drop than APP. LPS translocated from the digestive tract to portal blood are subject to a high clearance rate in the liver [9], which resulted in the drop of LPS concentration in the peripheral blood and likely in the reduction of possible differences.

The effect of PM in APP concentrations, especially SAA, is in agreement with its low concentration in neutrophils, since SAA influences the release and function of neutrophils during the acute phase response. The remaining blood variables were not affected by treatment, mainly because all the heifers were fed on the same high grain diet.

Conclusions

Both additives were successful in attenuating the reticular pH drop (time spent <5.6) compared to control in heifers fed a high grain based diet. Moreover the polyphenol based supplement was effective in limiting the acute phase response without interfering with DMI. Rumen, blood and feces LPS concentrations were not affected by dietary treatment probably due to wide variability and the mechanism of translocation and/or clearance in the liver. Further studies are needed to better understand the influence of tested compounds on the rumen microbial community and on dairy cow performance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions GM and IA designed the feeding trial which was conducted by RDN, GM, RR and SS. RDN and SL performed the chemical analyses. RDN, GM, KP, SL, EK and SS analyzed and interpreted the data, and drafted the article. All authors provide editorial content and have read and approved the final manuscript.

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CHAPTER 8

Effect of dicarboxylic acids and polyphenols on rumen microbial population in dairy heifers fed a high grain diet through metagenomic analysis

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Effect of dicarboxylic acids and polyphenols on rumen microbial population in dairy heifers fed a high grain diet though metagenomic analysis

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Abstract

Background: The aim of this study was to investigate the effects of two feed supplements on rumen bacterial communities of heifers fed a high grain diet. According to a Latin square design, six Holstein-Friesian heifers received one of the following dietary treatments: no supplement (control C), fumarate-malate (organic acid, O) (60 g/day) and polyphenol-essential oil (P) (100 g/day). Rumen fluid was analyzed to assess the microbial population using Illumina sequencing and quantitative real time PCR.

Results: Compared to the C and O treatments, the P treatment led to the highest number of observed species ($P < 0.10$), Chao1 index ($P < 0.05$), abundance based coverage estimated (ACE) ($P < 0.05$), and Fisher's alpha diversity ($P < 0.10$); whereas the O treatment had intermediate values between C and P with the exception of Chao1. PCoA

with unweighted Unifrac distance showed a separation among dietary treatments (P = 0.09) above all between C and P treatments (P = 0.05). The O and P treatments showed a significant increase of the family *Christenenellaceae* and a decline of *Prevotella brevis* compared to C. Additionally, P significantly enhanced the abundance of many taxa belonging to *Bacteroidetes*, *Firmicutes* and *Tenericutes* phyla due to antimicrobial activity of flavonoids that widened competition among bacteria.

Conclusions: Data suggest that both additives (organic acid and polyphenols) had effects on rumen bacterial populations during high-grain feeding in dairy heifers. Polyphenol treatment enhanced the rumen habitat with the increase of the microbial biodiversity promoting a competition among different taxa due to its antimicrobial activity.

Keywords: Ruminal acidosis, Rumen microbiota, Fumarate-malate, Polyphenol, Heifers

Background

The rumen is an anaerobic habitat and is composed of a large variety of microorganisms, including bacteria, protozoa, fungi and viruses. Among these microorganisms, bacteria play a critical role in ruminal fermentation and their function greatly affects the production and health of dairy cattle (Bevans et al., 2005; Fernando et al., 2010). High-grain diets used to increase the performance of dairy cows can disturb microbial communities in the rumen and the symbiosis between the host and these communities by causing the production of excessive amounts of organic acids (volatile organic acid (VFA) and lactate) (Plaizier, 2009; Zebeli et al. 2012; Marchesini et al., 2013). High concentrations of VFA and lactate can overcome the buffering capacity of the rumen resulting in major changes in the rumen environment, such as a pH depression, and impaired microbiota (Khafipour et al., 2009b; Fernando et al., 2010; Hook et al., 2011). The main bacterial phyla during high grain feeding are *Firmicutes*, and *Bacteroidetes*

(Khafipour et al., 2009b). The populations of starch-fermenting and lactic acid-utilizing bacteria also increase when high starch diets are fed (Nagaraja and Titgemeyer, 2007). An excessive depression in rumen pH causes a reduction in the number of cellulolytic bacteria (Russell and Wilson, 1996), major shifts in bacterial populations, and increases the lysis of Gram-negative bacteria resulting in the increase of free lipopolysaccharide (LPS) endotoxins (Plaizier et al., 2009; Plaizier et al., 2012; Mao et al., 2013). When the translocation of free LPS from the rumen and/or gut into portal ciculation occurs, an immune response in the host is induced. Moreover, the increase in proportion of bacteria such as *Enterobacteriacae* during high starch feeding increase the presence of virulence factors (fimbrial adhesins, heat-stable and heat-labile toxins and inflammatory peptides), which have the potential to cause inflammation (Plaizier et al., 2009; Khafipour et al., 2011; Plaizier et al., 2012).

Many nutritional strategies are used to avoid the onset of SARA in dairy cows, among which there are also the use of feed supplements to enhance the rumen microbial community and subsequently ruminal fermentation. These include the use of yeasts, probiotic bacteria (Ghorbani, et al., 2002; Bach et al., 2007; Long et al., 2014), and the addition of dicarboxylic acids (Nisbet et al., 2009), flavonoids (Balcells et al., 2012) as well as essential oils (Calsamiglia et al., 2007).

It has been suggested that the dicarboxylic acids malate and fumarate attenuate the ruminal pH drop during high grain feeding (Nisbet et al., 2009), and that they may increase the activity of the succinate-propionate metabolic pathway in several rumen bacteria, resulting in increased lactic acid uptake and production of propionate (Martin, 1998; Khafipour et al., 2009a). In *in vitro* rumen fermentative experiments, several essential oil (EO), or blends of EO have been demonstrated to enhance rumen fermentation (Calsamiglia et al., 2007; Marchesini et al., 2014). However, few *in vivo*

studies have investigated the effects of EO on rumen fermentation and bacterial populations (Benchaar et al., 2012; Zened et al., 2013). In addition, the effects of addition of polyphenolic compounds like flavonoids to the diet may include prevention of the pH reduction and the decrease of the acetate-to-propionate ratio due to the increase of the numbers of lactate-consuming and propionate-producing bacteria (e.g., *Megasphaera elsdenii*, *Selemonas ruminantium*) (Balcells et al., 2012).

This paper is a complementary part of a study (De Nardi et al., 2014) that investigated the effect of organic acid (O) and polyphenols (P) on reticular pH drop and acute phase response in dairy heifers fed a high grain diet. The aim of this study was to report the effects of the same supplements (O and P) on the rumen bacterial populations on high-grain feeding in dairy heifers, and to assess the mechanisms through which these supplement enhance rumen fermentation under this condition.

Materials and Methods

Animals, Experimental Design and Dietary Treatments

The experimental design and dietary treatments are described in De Nardi et al., 2014. Briefly, the study was carried out according to a 3 x 3 Latin square design, with 3 periods and 3 dietary treatments. During experimental periods that lasted 22 days, a low starch (LS) diet was given *ad libitum* to the heifers diet for 14 d, followed by feeding a high starch (HS) diet for 8 d (from d 15 to d 22). From the d 18 to the d 22, barley meal was gradually increased from 0.5 to 1.5 kg (250 g/day) to cause a rumen pH drop. During the HS diet, the six Holstein-Friesian heifers were offered one of the following three dietary treatments: i) no supplement, C treatment; ii) a daily dose of 60 g of fumarate-malate mixture, O treatment; iii) a daily dose of 100 g polyphenol-essential oil mixture, P treatment.

Sampling and analyses of ruminal fluid were also reported by De Nardi et al. (2014). Rumen fluid was transferred to 50-ml sterile tubes and immediately frozen in liquid nitrogen and then stored at -20°C until analysis for quantitative real-time PCR (qPCR) and Illumina sequencing.

DNA extraction

Rumen fluid samples were thawed at room temperature quickly and then kept on ice. One millilitre of rumen fluid was centrifuged at 15,000 × g followed by removing supernatant. DNA was extracted from pellets (200 mg of each sample) using ZR fecal DNA MiniPrep™ kit (Zymo Research Corp., Irvine, CA) which included a bead-beating step for the mechanical lysis of the microbial cells.

At the last step of the procedure, DNA was eluted from the column with elution buffer, and DNA concentration and purity were then determined spectrophotometrically by measuring the A260/280 (NanoDrop 2000, Thermo Scientific, DE, USA). The DNA in all samples was diluted with the same elution buffer to a final nominal concentration of 20 ng/µl and quality was checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. 2009b. Amplicons were verified by agarose gel electrophoresis (Khafipour et al., 2009b).

The DNA samples for qPCR analysis were diluted 2 ng/µl and aliquoted into 10 µL/vial, which is enough for testing one set of primers, in order to avoid repeated freeze-thaw cycles. All DNA samples were stored at -80°C until analysis.

Sequencing

Library Construction and Illumina Sequencing

The Library construction and Illumina sequencing were performed as described by Derakhshani et al. (2014, in press).

In brief, using modified F515/R806 primers (Caporaso et al., 2012) the V4 region of 16S rRNA gene was targeted for PCR amplification. The reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. Each sample was performed in duplicate in the PCR reaction and contained 1.0 µl of pre-normalized DNA, 1.0 µl of each forward and reverse primers (10 µM), 12 µl HPLC grade water (Fisher Scientific, ON, Canada) and 10 µl 5 Prime Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). After an initial denaturing step at 94°C for 3 min, 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec were performed with the aim of an extension step at 72°C for 10 min in an Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany).

ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) were used to purify the PCR products to remove primers, dNTPs and reaction components.

The pooling 200 ng of each sample was then used to generate the V4 library and quantified by Picogreen dsDNA (Invitrogen, NY, USA). After the procedure was multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, measured by Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). At the end with the aim to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries, 15% of PhiX control library was spiked into the amplicon pool. Customized sequencing primers for read1 (5´-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read2 (5´-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3´) were synthesized and purified

by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA). These were added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, CA, USA). A MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada were used to the 150 paired-end sequencing reaction.

Quantitative PCR analysis

Rumen fluids samples were analyzed using the qPCR using the primers for *Eubacteria*, *Prevotella brevis*, *Prevotella bryantii*, *Fibrobacter succinogenes*, *Megasphaera elsdenii*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, *Lactobacillus spp*., *Streptococcus bovis* described in Wang et al. (1997), Ozutsumi et al. (2006), Denman and McSweeney (2006), Khafipour et al. (2009b), Fernando et al. (2010). The oligonucleotides were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada). Real-time PCR was carried out using a CFX connect Real Time system (Bio-Rad Laboratories, Inc., USA). Each reaction mixture was run in triplicate in a volume of 15 µl in optical reaction plates (Thermo Fisher Scientific, U.K.) sealed with optical adhesive film (Applied Biosystems, Foster City, CA, USA). Amplification reactions were carried out with 7.5 µl Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) mixed with the selected primer set at a concentration of 0.5 µM for each primer and 10 ng of genomic DNA. Amplification consisted of one cycle of 95°C (10 min), 40 cycles of denaturation at 95°C (15 s), and annealing/extension at 60°C (1 min). Final melting analysis was obtained by slow heating from 65°C to 95°C in order to assess the specificity of the primer set. Data were normalized for *Eubacteria* using the universal bacteria 16S RNA gene primer sets, which detect all bacterial strains.

Bioinformatic Analyses

Bioinformatic analyses were performed as described by Derakhshani et al. (2014, in press). In brief, with the aim to merge overlapping paired-end Illumina fastq files the PANDAseq assembler (Masella et al., 2012) was used. We discarded all the sequences with mismatches or ambiguous calls in the overlapping region. Downstream computational pipelines of the open source software package QIIME (Caporaso et al., 2010a) analyzed the output fastq file was then analyzed by. According to the barcode sequences, assembled reads were demultiplexed and exposed to additional quality-filters and reads with more than 3 consecutive bases with quality scores below 1e-5 were truncated, and those with a read length shorter than 75 bases were removed from the downstream analysis. UCHIME (Edgar et al., 2011) was used to filter chimeric reads and using the QIIME implementation of UCLUST (Edgar et al., 2010) at 97% pairwise identity threshold the sequences were assigned to Operational Taxonomic Units (OTU). RDP classifier (Wang et al., 2007) was used to assign taxonomies to the representative sequence of each OTU and using PyNAST algorithms (Caporaso et al., 2010b), aligned with the Greengenes Core reference database (DeSantis et al., 2006). Chao1 (Chao 1984; Colwell and Coddington 1994), abundance based coverage estimated (ACE) (Chazdon, et al., 1998), Shannon and Simpson indices are calculated using Phyloseq package (version 1.9.2) in R (version 3.0.2).

After verifying the normality of residual (PROC UNIVARIATE) of the observed species, richness, diversity and qPCR microbial variables were analyzed using the MIXED procedure with a compound symmetry structure along a linear model that included the fixed effects dietary treatment, period and their interaction, and the random effect of heifer. If significant dietary treatment effects were detected ($P < 0.10$), the LS*means* were

compared using the probability of differences (PDIFF) option and the Tukey adjustment test. To obtain a normal distribution and homogeneous residual error, Simpson (diversity) and qPCR microbial variables were log transformed (SAS, release 9.3, 2010).

β-diversity indices (Bray-Curtis and unweighted Unifrac) were submitted to distance-based permutational multivariate analyses of variance (PERMANOVA, Anderson, 2005) to test the effect of dietary treatments followed by pair-wise comparison among their levels using Primer (version 6.0, PRIMER-E Ltd, Plymouth, UK).

Non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) were conducted to evaluate differences in community structure among dietary treatments (β-diversity). NMDS plots were generated using Bray–Curtis distance (Bray and Curtis, 1957) and PCoA using unweighted Unifrac distance (Lozupone, et al., 2006) (R package vegan version 2.0-10, 2013).

At bacterial taxa, pair-wise differential expression analysis among dietary treatments were performed in the nbinomWaldTest method of DESeq2 (Love, 2014). The statistical model for differential expression analysis with DESeq2 consisted of the effects of dietary treatment, period and heifer; these were considered significant at $P < 0.05$ and trends were discussed at $0.05 < P < 0.10$ (DESeq2, R package version 1.5.34, 2012).

Results

Illumina sequencing produced 317,369 sequences across treatments. The average numbers of sequences were 16,413, 18,814 and 28,247 for the C, O and P treatment, respectively. The number of the generated sequences were not affected by dietary treatment ($P = 0.60$).

Regarding richness indices, heifers on the P treatment tended to have the highest number of observed species ($P < 0.10$) and ACE ($P < 0.05$), whereas the O treatment had intermediate values between C and P in the number of observed species and ACE (Table

1). The Chao1 index was higher in the P treatment ($P < 0.05$) compared to the C and O treatments. Moreover, Fisher's alpha diversity tended ($P < 0.10$) to be higher in heifers on the P treatment compared to those on the C treatment, whereas the O treatment resulted in intermediate values. Shannon and Simpson indices were not affected by treatments.

PERMANOVA analisys showed a significant ($P < 0.10$) significant effect of dietary treatments both based on Bray-Curtis distance and unweighted Unifrac distance (Table 2). Using the Bray-Curtis and unweighted Unifrac distances analyses, differences were observed in bacterial population among periods ($P = 0.02$ and $P = 0.06$, respectively). Animal also varied in the dissimilarities measurements, particularly in unweighted Unifrac ($P = 0.09$). The multiple-comparisons based on Bray-Curtis distance showed only a tendency ($P = 0.11$) towards a significant difference between dietary treatments (C vs P), while the multivariate analysis based on uniweightd Unifrac distance showed a significant ($P = 0.05$) separation between the C and P treatments (Table 2). Comparison of the bacterial communities by non-metric multidimensional scaling (NMDS) with Bray-Curtis distance (Figure 1) and principal coordinate analysis (PCoA) with unweighted Unifrac distance (Figure 2) confirmed a low separation among dietary treatments.

The abundance of the 20 phyla detected by using Illumina was not affected by dietary treatments. Across treatments among these four bacterial phyla (*Bacteroidetes*, *Firmicutes, Tenericutes* and *Euryarchaeota)* were abundant (> 1%) and accounted for 94.8% of total bacterial community. *Spirochaetes*, *Proteobacteria*, *Actinobacteria*, *Fibrobacteres*, *Chloroflexi*, *Cyanobacteria* and *Verrucomicrobia* were in low abundance (0.1-1%), and unclassified bacteria accounted for 2.3%.

Illumina sequencing detected 32 classes, 55 orders, 87 families and 88 genera. The predominant sequences (>1%) within *Bacteroidetes* belong to order *Bacteroidales* (22.71%), genus *Prevotella* (21.17%), genus *Paludibacter* (2.24%), family *BS11* (2.04%),

family *S24-7* (1.77%), and genus *CF231* (1.06%). The predominant sequences in *Firmicutes* were genus *Butyrivibrio* (4.30%), and family *Christensenellaceae* (2.43%), and in phylum *Tenericutes* they were order *RF39* (1.74%). In particular, the cellulolytic bacteria *Ruminococcus*, *Treponema* and *Fibrobacter* represented 8.15%, 0.58% and 0.19%, respectively on average of total bacteria. The relative abundance of the starch-fermenting bacteria were low and included: *Bifidobacterium* (0.43%), *Eubacterium* (0.03%), *Selenomonas* (0.02%), *Succinivibrio* (0.02%), and *Ruminobacter* (<0.01%). The relative abundance of *Butyrivibrio* (4.30%) was high, while *Succimonas*, *Lactobacillus* and *Streptococcus* were not detected. Among lactic acid-utilizing bacteria, *Anaerovibrio* (0.01%) was detected, whilst *Fusobacterium* (0.00%) was not detected. The statistical analyses of these data (OTUs –Illumina), referred to the single taxa, carried out by using the negative binomial model (Deseq) showed a total of 19 significant pair- wise comparisons (O vs C, P vs C, and P vs O) as reported in figure 3. The comparison between O and C highlighted that only the family *Christensenellaceae* was significantly ($P < 0.05$) more abundant higher in O (Figure 3a). The major number of OTUs affected by treatments were observed in comparison between the P and C treatments (11 OTUs). In particular, P dietary treatment showed significant higher OTUs in the case of family *BS11* (phylum *Bacteroidetes*), genus YRC22 (phylum Bacteroidetes), genus *CF231* (phylum *Bacteroidetes*), phylum *Bacteroidetes*, order *Bacteroidales* (phylum *Bacteroidetes*), genus *Paludibacter* (phylum *Bacteroidetes*), genus *Butyrivibrio* (phylum *Firmicutes*), family *Christensenellaceae* (phylum *Firmicutes*), family *Mycoplasmataceae* (phylum *Tenericutes*), and genus RFN20 (phylum Tenericutes). The contrary occurred for thefamily *WCHB1-25* (phylum *Verrucomicrobia*) (Figure 3b). Moreover, the P treatment was significantly increased the abundance of genera *YRC22*, *CF231*, *RFN20*, and *Anaeroplasma* (phylum *Tenericutes*) compared to the O treatment; the contrary for order

Actinomycetales (phylum *Actinobacteri*a), the family *Pseudomonadaceae* (phylum *Proteobacteria*) and family WCHB1-25 (Figure 3c).

The comparison of single species based with qPCR and submitted to the linear MIXED model evidenced a significant effect of the dietary treatments only in the case of *Prevotella brevis.* Particularly, this species was higher (P = 0.03) in the C treatment compared to the other treatments (Figure 4).

Discussion

This study was conducted to investigate the effects of the feed supplements, organic dicarboxylic acids (O) and polyphenols (P), on the rumen bacterial biodiversity in dairy heifers fed a high-grain diet. The effects of these additives on reticular pH and immune response was investigated and published earlier by De Nardi et al., (2014), in which both additives, O and P, demonstrated to attenuate the reticular pH drop (time spent < 5.6) due to high-grain feeding. In particular P resulted to be more effective to reduce the inflammatory response without interfering with DMI.

The effects of supplements on ruminal microbial community, the higher richness and diversity found in the P treatment, compared with control, suggest a positive effect of the P supplement on microbial population. This is in agreement with other studies (Khafipour et al., 2009b; Fernando et al., 2010; Zened et al., 2013) which report a higher richness and diversity for cows with physiological rumen pH and rumen function compared to cows with impaired rumen parameters due to high-grain feeding. In part this could be explained by the shorter time spent by P group under the reticulo-ruminal pH of 5.6 (as shown in De Nardi et al., 2014), which did not compromise the bacterial population. Despite the O treatment showing a similar effect on reticulo-ruminal pH, compared to P, on the time spent daily below pH 5.6 (De Nardi et al., 2014), it did not lead to a similar effect on

richness and diversity as observed in P. This difference could be explained by mechanisms of action through which the supplements affect microbial population. The organic acid supplement seems may offer electron sink for H_2 allowing an increase of lactate utilization by bacteria which use the succinate-propionate pathway to synthesize succinate and (or) propionate (Martin, 1998; Nisbet et al., 2009). The blend containing flavonoids, i.e. polyphenols, thanks to its antimicrobial activity (Harborne and Williams, 2000; López-Lázaro 2009) seems to promote bacteria that metabolize lactate to VFA avoiding lactate accumulation in the rumen (Balcells et al., 2012).

Illumina sequencing highlighted significant effects of dietary treatments on some phyla, orders, families, and genera, while qPCR found significant differences among treatments only for *Prevotella brevis*. Although the supplements led to different in their effects on microbial population they both significantly led to an increase of abundance of the family *Christensenellaceae* and a drop of *Prevotella brevis* compared to C. This is probably due to the attenuation of pH drop. This is in line with what found by Morotomi et al. (2012), who reported that the *Christensenellaceae* grow between pH 6 to 9. Moreover, Matsui et al. (2000), reported that *Prevotella spp.* are involved in starch degradation and utilization and thus grow better at low pH conditions as reported by Khafipour et al. (2009b).

On the other hand compared to the O treatment, the P treatment led to a decrease of family *Pseudomonadaceae*, order *Actinomycetales* and a family belonging to subdivision of *Verrucomicrobia*. This reduction is probably due to a potential antimicrobial activity of flavonoids as reported in literature (López-Lázaro 2009; Balcells et al. 2012). The decrease of these microbials seemed to favor the increase of several genera belonging to *Bacteroidetes* and *Tenericutes*. Compared to control, the P treatment led to the same trend seen before with the addition of the increase of some *Firmicutes* such as genus *Butyrivibrio*.

The higher pH and the antimicrobial activity towards some microbial families observed in the P treatment allowed an increase of bacteria which usually grow better in cows fed low starch diet and with a higher reticular pH. These include bacteria belonging to the family *BS11* as reported by Zened et al. (2013) and *Paludibacter* (Pitta et al., 2010); the latter was found to decrease when steers were transitioned from a hay-based to cereal grain-based diet. Even genus *Butyrivibrio* showed the same behavior increasing in higher pH condition since it is usually involved in fibre degradation (Krause et al., 2003).

Conclusions

Both additives (organic acid and polyphenols) tested led to significant microbial changes in microbial communities in the rumen, compared to control diet that are in line with the reticulo-rumen pH variations obtained. Polyphenols strongly increased the microbial biodiversity in terms of richness and diversity more than organic acids. These different biodiversity patterns led to a discrimination among dietary treatments by using principal coordinate analysis. The increase in α diversity indicated the benefit of P supplement.
The differences in microbial composition in the rumen between organic acid and polyphenols were likely due to an antimicrobial activity of the latter that widened the competition among different taxa.

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Table 1. Number of observed species, richness (Chao1 and ACE) and diversity estimators (Shannon, Simpson and Fisher'alfa) in control, organic acid and polyphenols dietary treatments

	Observed species	Richness[1]		Diversity		
		Chao1	ACE	Shannon	Simpson[2]	Fisher's alpha[3]
Control	2,116$^{\beta}$	3,420^{b}	3,731^{b}	5.9	0.99	645$^{\beta}$
Organic acid	2,255$^{\alpha\beta}$	3,834^{b}	4,214^{ab}	6.1	0.99	691$^{\alpha\beta}$
Polyphenols	2,742$^{\alpha}$	7,164^{a}	6,927^{a}	6.1	0.98	890$^{\alpha}$
SEM	188.7	653.1	711.8	0.22	(0.99-0.98)	78.2
P-value	0.08	0.03	0.06	0.90	0.93	0.07

[1]Based on Chao1 richness indices.
[2]Statistical analysis was conducted on natural logarithm (ln) transformed data that are presented as ln back transformed and 95%-confidence interval in brackets.
[3]Based on Fisher's alpha diversity estimators.
ACE, abundance based coverage estimated.
$^{a,\ b}$Within column indicate statistical differences at P-value < 0.05; $^{\alpha,\ \beta}$Within column indicate statistical differences at P-value < 0.10.

Table 2. **PERMANOVA analysis of the effect of dietary treatments, periods and heifers on rumen bacteria dissimilarities based on Bray-Curtis and unweighted Unifrac**

Source	Bray-Curtis		Unweighted Unifrac	
	pseudo-*F*	*P*-value	pseudo-*F*	*P*-value
Dietary treatment	1.208	0.10	1.155	0.09
Period	1.328	0.02	1.174	0.06
Heifer	1.175	0.11	1.135	0.09
Pair-wise tests				
Control vs Organic acid		0.14		0.22
Organic acid vs Polyphenols		0.30		0.15
Control vs Polyphenols		0.12		0.05

P-values were calculated on 999 possible permutations.

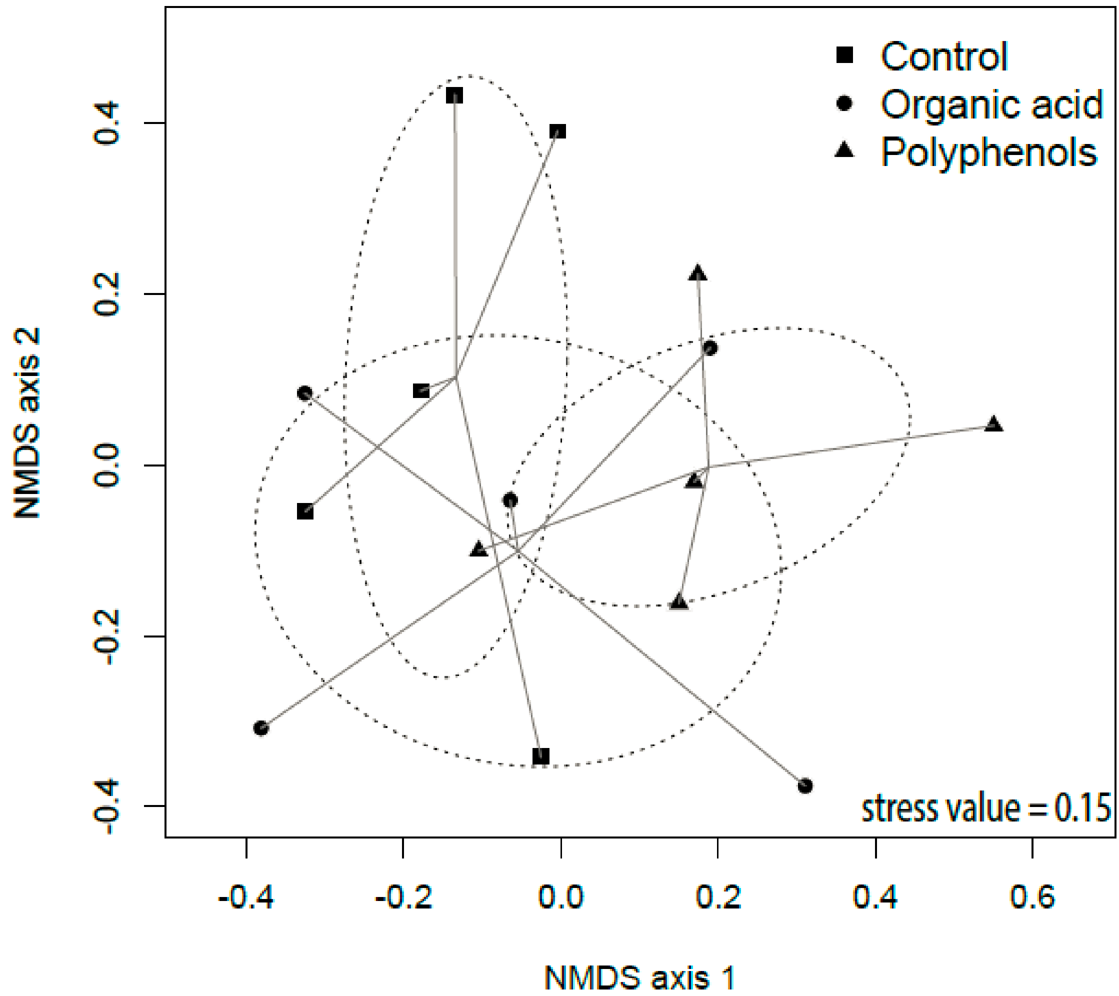


Figure 1. Non-metric multidimensional scaling (NMDS) using Bray-Curtis to explore dissimilarities in microbial composition among dietary treatments. Stress value indicates the reliability of test.

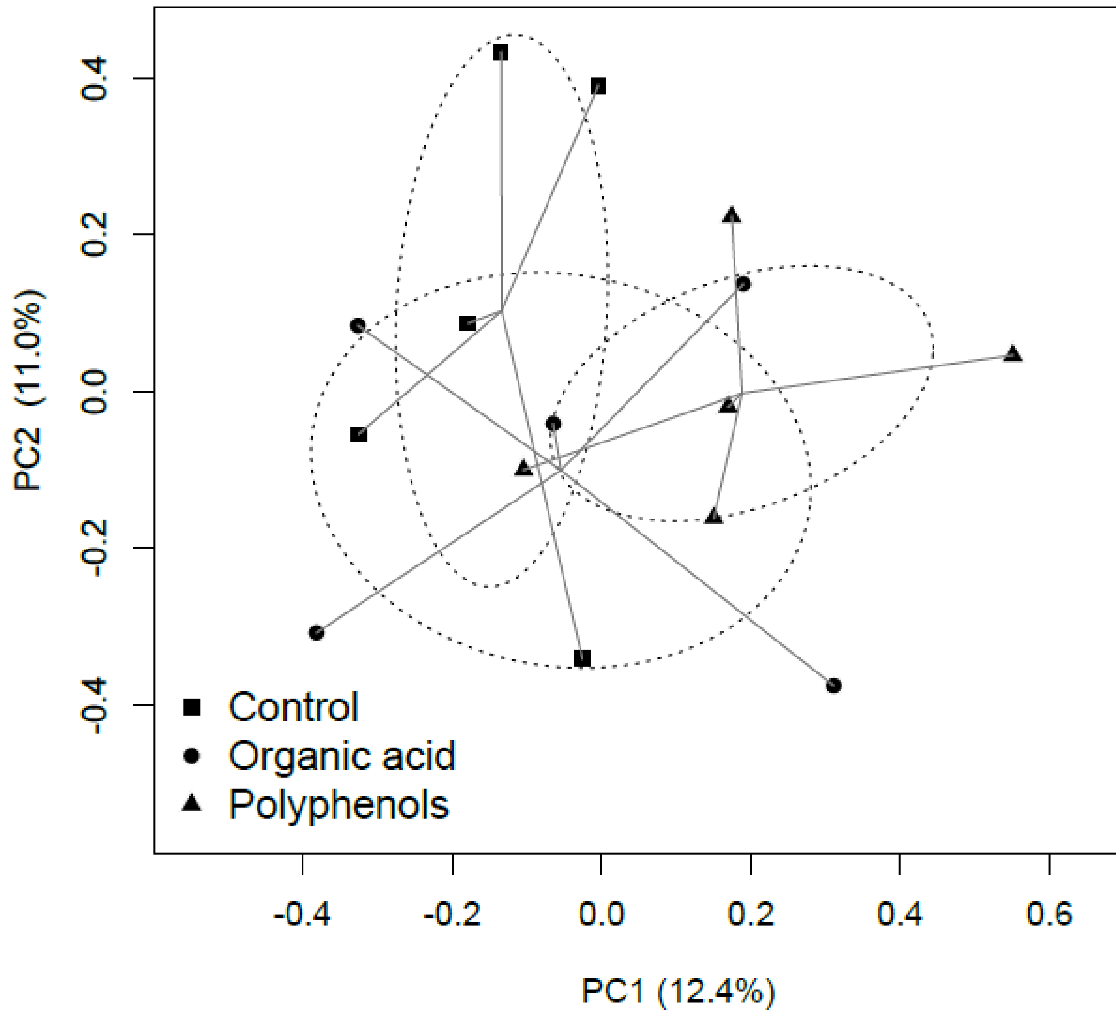
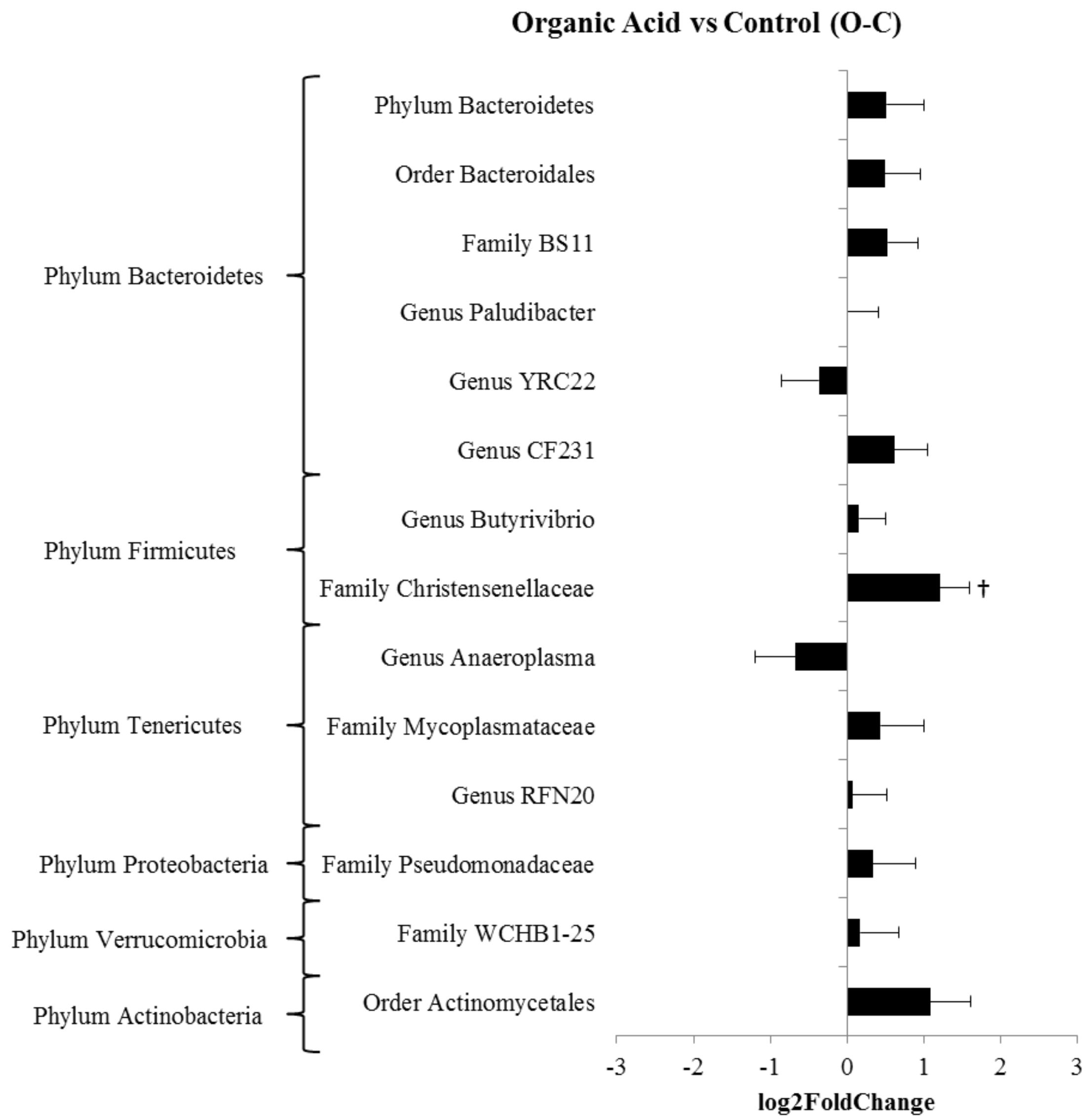
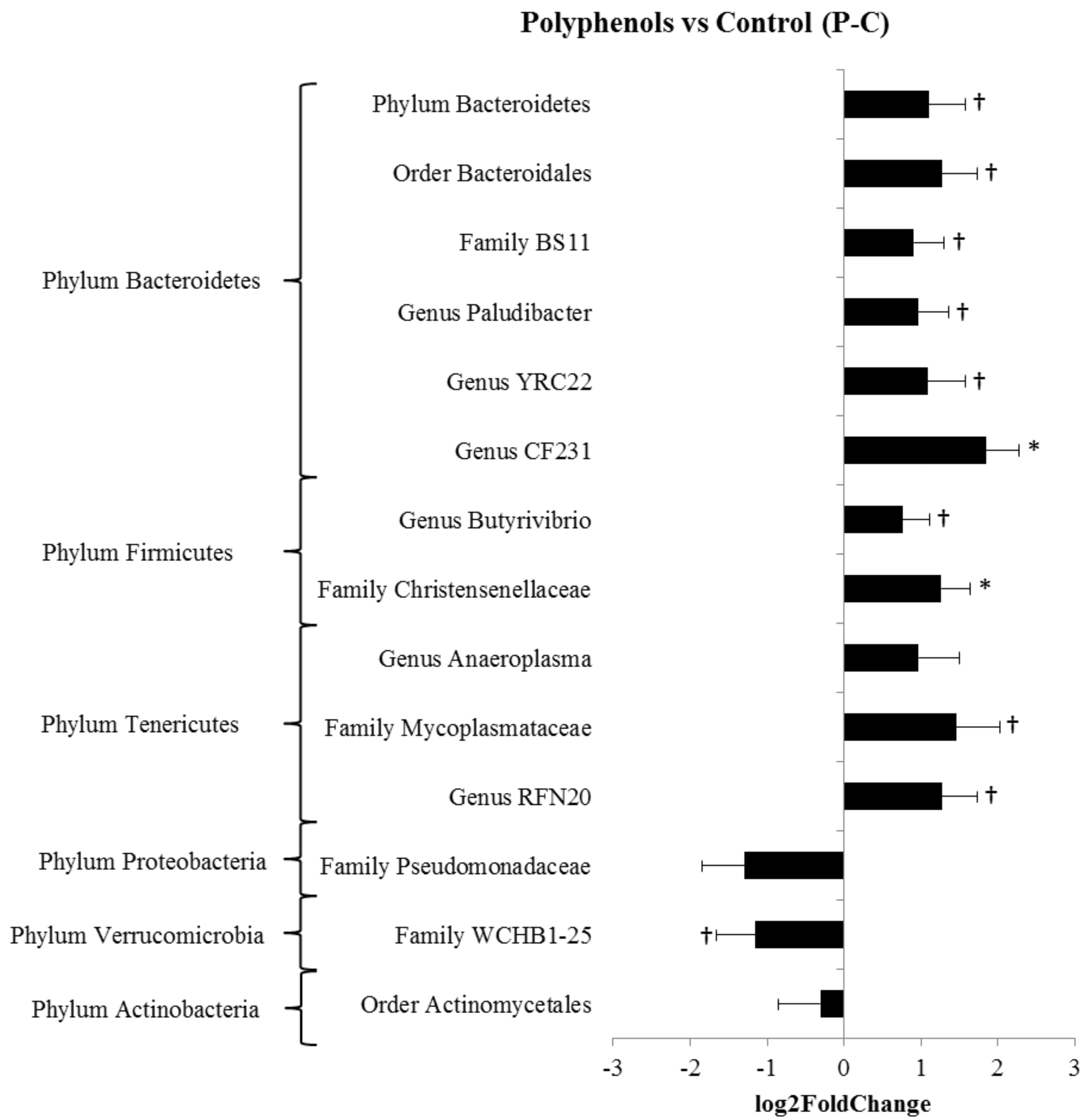


Figure 2. Principal coordinate analysis (PCoA) using unweighted Unifrac to explore dissimilarities in microbial composition among dietary treatments. The axes (PC1 = 12.4% and PC2 = 11.0%) account for 23.4% of the total variation of the model.

a)



b)



c)

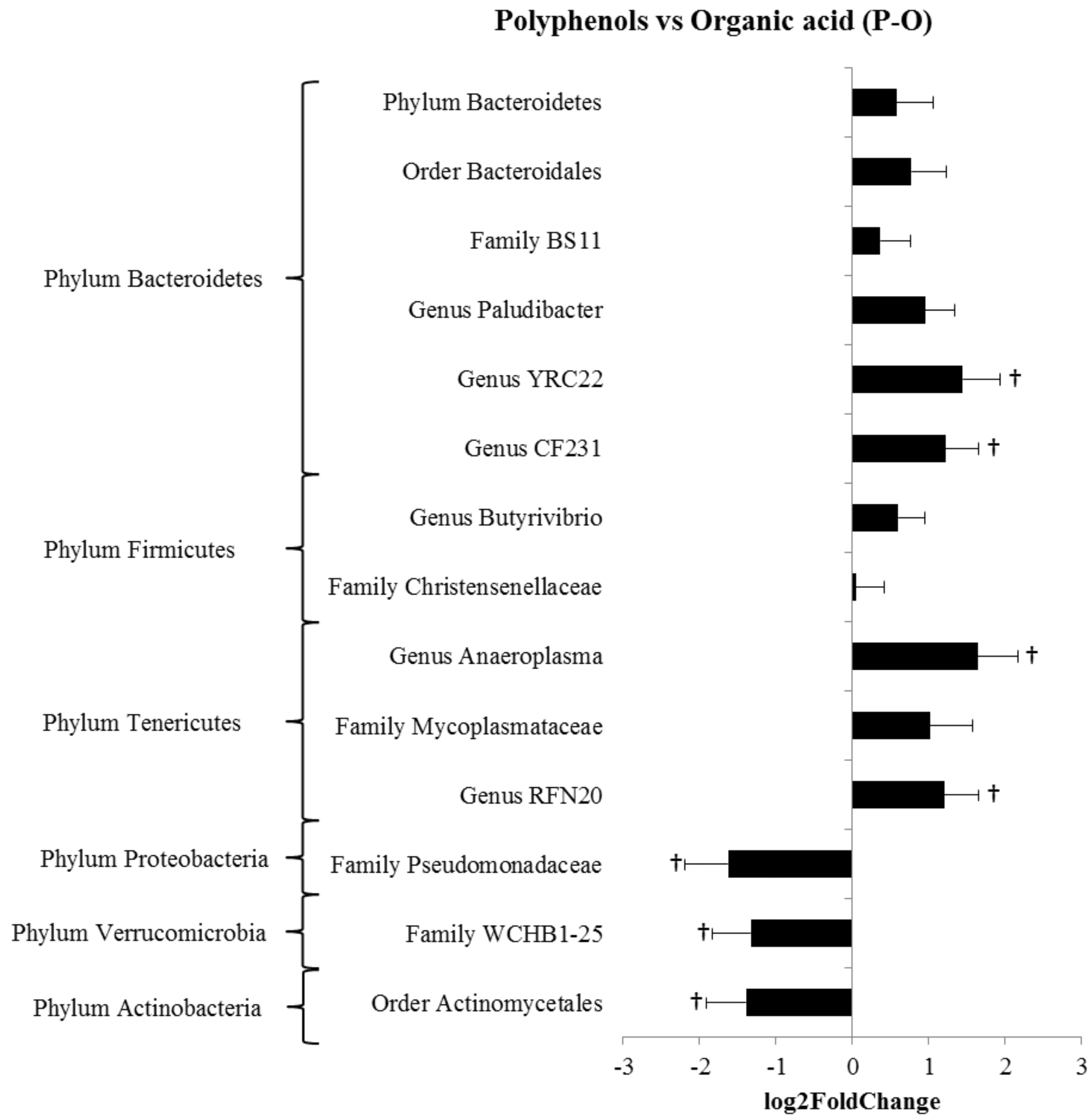


Figure 3 a, b, c. Changes ($\log_2$) in rumen microrganisms in the comparison of a) organic acid vs control, b) polyphenols vs control, and c) polyphenols vs organic acid determinated with Illumina.

†: $P < 0.1$; *: $P < 0.05$. Bar = standard error.

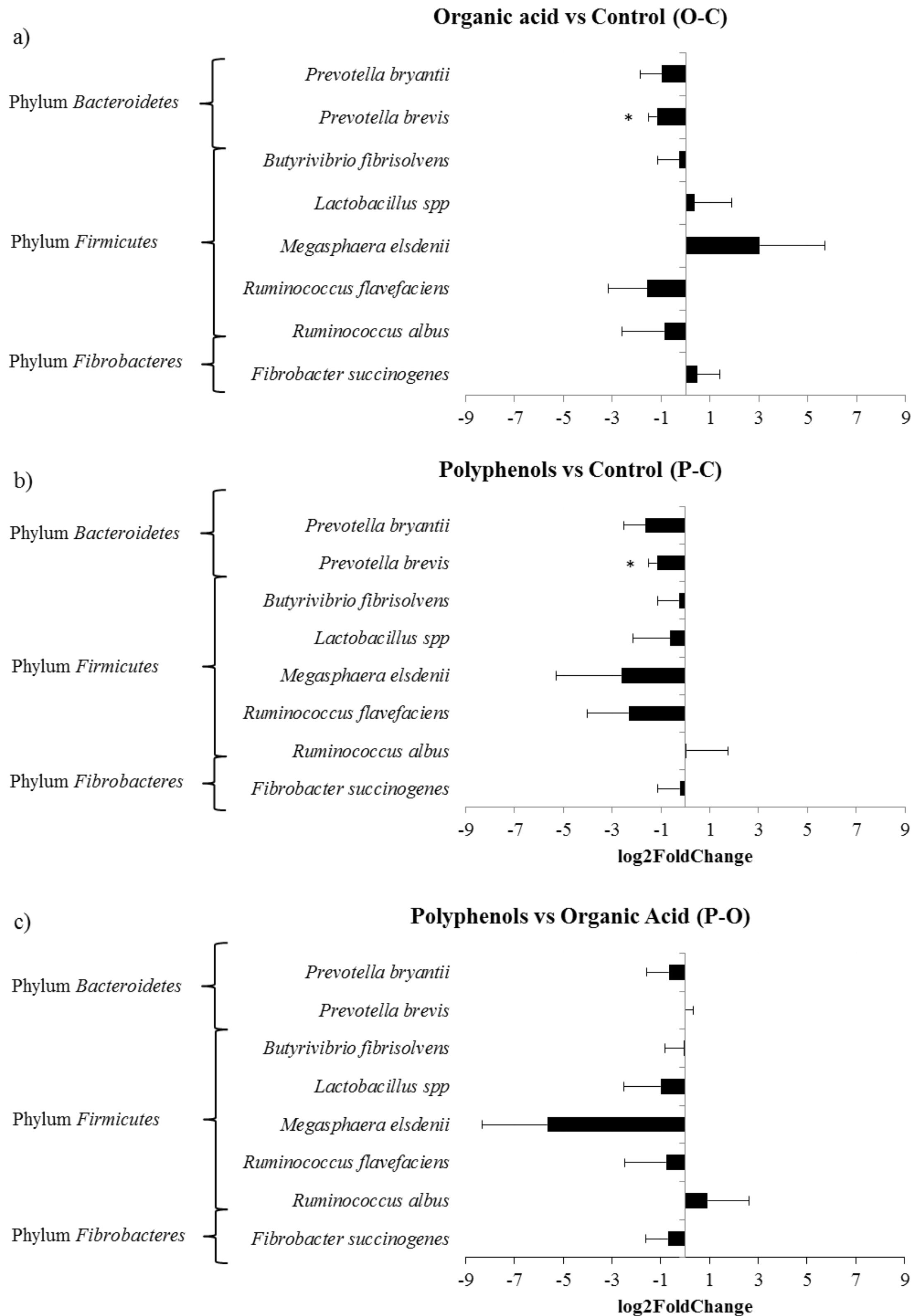


Figure 4. Changes (log2 fold) in predominant rumen microorganisms in the comparison of a) organic acid vs control, b) polyphenols vs control, and c) polyphenols vs organic acid determined with quantitative real-time PCR. * < 0.05; bar = standard error.

CHAPTER 9: General conclusions

This research focused on diagnosis and prevention measures of SARA in dairy cows, which result fundamental for the reduction of direct and indirect costs in the management of intensive dairy farms. As regards, the diagnosis of SARA, both the direct and indirect methods tested, such as rumen boluses and blood variable screening may be easily used by farmers in intensive dairy farms and, combined with the use of good management practices and nutritional strategies, can help to prevent the onset of ruminal acidosis.

Rumen and reticular boluses allow to record ruminal variables (temperature, pH, etc.) continuously, to assess the ruminal status and evaluate rumen health in dairy farms with the aim of improving animal performance and welfare and permitting to quickly make the appropriate adjustments in feeding and management practices to reduce the incidence of SARA. Moreover boluses resulted more sensitive in the diagnosis of SARA than rumenocentesis, although results of pH measures showed good correlation between the two methods. The limit in the field use of rumen boluses by farmers is so far represented by relatively high costs and short battery life, two aspects that should be improved to allow the spread of this technology.

Blood variables, like acute-phase proteins (APP), are well known to be useful in detecting inflammation and in this research their values together with those of other variables changed between healthy heifers and heifers with SARA. Although in this research the increase of APP was attributed to SARA because non pregnant heifers were used and animals were checked to exclude other causes of inflammation, in general the raise of these variables alone is to be considered a non-pathognomonic

sign. However, the best approach to diagnose SARA from blood variables seems to be a multifactorial approach. In this research the use of 5 variables (haemoglobin, mean platelet volume, β-hydroxybutyrate, glucose and reduced haemoglobin) through a canonical discriminant analysis (CDA) allowed to discriminate between healthy animals and animals affected by subacute or acute ruminal acidosis. Animals affected by very light forms of SARA (risk of SARA) were anyhow confused with healthy animals.

The nutritional strategies tested to prevent the onset of SARA in high grain feeding conditions confirmed that both the physical form of the ration and the presence of certain supplements is useful to modulate the risk of rumen acidosis. As well as the fibre (NDF) particle length, also the length of the cereal particles resulted important in determining the risk of SARA. Maize particles shorter than 1 mm indeed favored an increase of milk protein, but decreased the ruminal pH. Among the supplements tested, although many of them showed some influence on rumen fermentations (*in vitro*), only dicarboxylic acids and polyphenols resulted in the prevention of the drop of ruminal pH in high grain feeding conditions. Polyphenols were more effective in limiting the acute phase response and significantly increased the microbial biodiversity in terms of richness and diversity. The differences in microbial composition in the rumen between organic acids and polyphenols were likely due to an antimicrobial activity of the latter that widened the competition among different taxa.

Summarazing, the use of supplements such as polyphenols seem to allow the ruminal pH to remain within a physiological range, reducing the effects of SARA on rumen fermentations, improving animal welfare, productivity and profitability with the advantage of easy managment by farmers without time of suspension.

Highlights

- Rumen-floating and reticular boluses resulted more sensitive in the diagnosis of SARA than rumenocentesis, although results of pH measures showed positive correlation between the two methods.
- Some blood variables like LBP and other acute-phase proteins appear to be useful in detecting SARA, although the best approach to diagnose SARA from blood variables seems to be a multifactorial approach.
- Feed particles dimensions, and in particular those related to cereal particles, resulted important to modulate both the quality of milk (protein concentration) and the risk of SARA.
- Dicarboxylic acids (fumarate-malate) and polyphenols resulted helpful in preventing the onset of SARA in high grain feeding condition and polyphenols reduced the inflammation response consequent to the translocation of LPS from the digestive tract to blood.
- Polyphenols increased the rumen microbial biodiversity in terms of richness and diversity in high grain feeding condition.

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