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 Actinomycetes bacteria and miglitol, a pseudomonosaccharide derivative of 1-deoxynojirymycin, 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).

Materials and methods

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, powder and extracted in isopropanol. Isopropanol was added to the flask 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 50-mL 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 carboxydase 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, Alzella, Spain) and 8 mL of each sample was collected for the measurement of VFA and ammonia N. Subsequently, 2 mL of m-phosphoric 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-μm Phenex-RC filters (Phenomenex Sh, 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 9A Shimadzu pump, a SIL 10A auto sampler and an RID-model Shimadzu10A detector (Shimadzu, Kyoto, Japan). Volatile fatty acids 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 of VFA, a 30-min isocratic program was run with 0.25 N H2SO4 as the mobile phase and 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 c-amylase and sodium sulphite, using an Ankom22© 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):

$$DMD = \frac{(substrate\ DM + inulin\ DM - residual\ DM)}{(substrate\ DM)}$$

where substrate is represented by maize meal and supplements, the inulin 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). LSmeans 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 |
| CP, g/kg DM       | 99         | 19          | 3           | 212         | 2    |
| EE, 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 carbohydrates 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 LSmeans 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 LSmeans 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 h | 15 mg | 100 mg | 24 h | 15 mg | 100 mg |
|----------|-----|-------|--------|------|-------|--------|
| S        | **  |       | **     |     | **    |        |
| BBE      | 6.23a | 5.83ab | 5.54ab | 6.16bc | 5.16bc |
| PHA      | 6.23a | 6.09ab | 5.57ab | 5.44ab | 5.44ab |
| CFL      | 6.11a | 6.07ab | 5.50ab | 5.53ab | 5.53ab |
| WMB      | 6.09a | 5.95ab | 5.49ab | 5.33bc | 5.33bc |
| ACB      | 6.52a | 6.52ab | 6.40ab | 6.27ab | 6.27ab |

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 h | 15 mg | 100 mg | 24 h | 15 mg | 100 mg |
|------------|-----|-------|--------|------|-------|--------|
| S          | **  |       | **     |     | **    |        |
| BBE        | 4.70a | 5.47a | 7.10b  | 7.90b |
| PHA        | 4.79a | 5.29a | 6.94b  | 7.50b |
| CFL        | 5.21a | 5.43a | 7.67a  | 8.14a |
| WMB        | 5.25a | 5.61a | 7.80a  | 8.01a |
| ACB        | 3.70b | 3.79b | 4.93b  | 5.31b |

S, supplement; BBE, bilberry extract from Vaccinium myrtillus; PHA, phaseolin extrac 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. **Within incubation time, different lowercase superscripts in the same row denote significant differences (P<0.05) with a dose of 15 or 150 mg. **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.
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-glucosidase 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, phytoestrogens and specific plant toxins-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 h         | 24 h        |
| 15 mg   | 150 mg      | 15 mg       | 150 mg      |
| SS      |             |             |
| BBE     | 2           | 2           | 107         | 28          |
| PHA     | 9           | 3           | 121         | 159         |
| CFL     | 25          | 73          | 234         | 273         |
| WMB     | 5           | 23          | 210         | 203         |
| ACB     | 72          | 56          | 345         | 392         |
| SEM     | 16.7        | 43.7        |
| P       |             |             |
| S       | *           | *           |
| D       | ns          | ns          |
| S×D     | ns          | ns          |

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 (±2.8) and 18 (±0.85) 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<0.05; †P<0.01; 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 h                        | 24 h                      |
| 15 mg   | 150 mg                     | 15 mg                     | 150 mg                     |
| S       |                            |                           |
| BBE     | 51.2₄                      | 49.0₈                     | 49.1₈                      | 47.8₈                     |
| PHA     | 51.5₄                      | 51.9₄                     | 49.1₈                      | 50.0₉₈                     |
| CFL     | 53.₂₄                      | 59.0₄₈                    | 50.0₈₉₈                    | 47.8₈₈                     |
| WMB     | 52.2₄₈                     | 52.7₉₈₈                   | 50.1₉₈₈₈                   | 50.0₉₈₈₈                   |
| ACB     | 54.1₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈₈¢\line(0,1)][Ital J Anim Sci vol.13:2014] [page 617]
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 and Russell, 1996; Szumacher-Strabel and Ciesłak, 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 carbohydrate 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 Control, failed to inhibit bacterial carbohydrolases 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/glucoamylase 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.

| Supplement | 5 h 15 mg | 5 h 150 mg | 24 h 15 mg | 24 h 150 mg |
|------------|----------|-----------|------------|------------|
| BBE        | 29.1±     | 32.7±     | 29.1±      | 33.4±      |
| PHA        | 28.3±     | 29.8±     | 28.3±      | 29.5±      |
| CFL        | 28.0±     | 29.7±     | 29.0±      | 29.7±      |
| WMB        | 29.8±     | 29.4±     | 28.9±      | 30.0±      |
| ACB        | 27.4±     | 26.3±     | 26.5±      | 28.6±      |
| SEM        | 0.70      | 1.04      |            |            |

P, S, BBE, PHA, CFL, WMB, ACB; 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 (±SE) 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.

| Supplement | 5 h 15 mg | 5 h 150 mg | 24 h 15 mg | 24 h 150 mg |
|------------|----------|-----------|------------|------------|
| BBE        | 0.35±     | 0.35±     | 0.68±      | 0.71±      |
| PHA        | 0.33±     | 0.33±     | 0.65±      | 0.64±      |
| CFL        | 0.33±     | 0.33±     | 0.66±      | 0.66±      |
| WMB        | 0.33±     | 0.38±     | 0.67±      | 0.69±      |
| ACB        | 0.26±     | 0.28±     | 0.57±      | 0.55±      |
| SEM        | 0.013     | 0.046     |            |            |

P, S, BBE, PHA, CFL, WMB, ACB; D, dose. **Within incubation time, different lowercase superscripts in the same row denote significant differences (P<0.05) with a dose of 15 or 150 mg. **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 an average value (±SE) of 0.34(±0.07) and 0.64(±0.12) at 5 and 24 h, respectively. *P<0.05; †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.

|                | 5 h (SEM) | 24 h (SEM) |
|----------------|-----------|------------|
|                | 5 mg      | 15 mg      | 15 mg      | 150 mg     | 15 mg      | 15 mg      | 15 mg      | 150 mg     |
|                |           |            |            |            |            |            |            |            |
| S              | 0.43aA    | 0.26aA     | 0.76aA     | 0.477a     |
| BBE            |           |            |            |            |            |            |            |            |
| PHA            | 0.41aA    | 0.29aA     | 0.76aA     | 0.70aA     |
| CFL            | 0.26aA    | 0.33aA     | 0.63aA     | 0.67aA     |
| WMB            | 0.28aA    | 0.12aA     | 0.72aA     | 0.65aA     |
| ACB            | nd        | nd         | nd         | nd         |
| SEM            | 0.047     | 0.036      |
| P              |           |            |            |            |            |            |            |            |
| S              | ns        |            |            |            |            |            |            |            |
| D              |           |            |            |            |            |            |            |            |
| SxD            |           |            |            |            |            |            |            |            |

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. *Within incubation time, different lowercase superscripts in the same row denote significant differences (P<0.05) with a dose of 15 or 150 mg. **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 an average value (±SD) of 0.38±0.11 and 0.73±0.07 at 5 and 24 h, respectively. *P<0.05; ns, not significant.

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[Ital J Anim Sci vol.13:2014] [page 619]