ABSTRACT: To study the effects of an extract of plant flavonoids (Bioflavex (FL)) in cattle fed high-concentrate diets, 2 experiments were designed. In the first experiment, the effects of Bioflavex on the development of rumen acidosis was evaluated in 8 Holstein-Friesian crossbreed heifers (451 kg; SEM 14.3 kg of BW) using a crossover design. Each experimental period lasted 22 d; from d 1 to 20, the animals were fed rye grass, on d 21 the animals were fasted, and on d 22, rumen acidosis was induced by applying 5 kg of wheat without [Control: (CTR) heifers who did not receive Bioflavex] or with flavonoids [heifers who received FL; 300 mg/kg DM] through a rumen cannula. Rumen pH was recorded continuously (from d 19 to d 22). On d 22, average rumen pH was significantly ($P < 0.01$) higher in the FL animals (6.29; SEM = 0.031) than it was in the CTR heifers (5.98; SEM = 0.029). After the wheat application, the rumen VFA concentration increased ($P < 0.01$), the proportion of acetic acid decreased ($P < 0.01$), and lactate concentration (mmol/L) increased, but the increase was not as great ($P = 0.09$) in the FL as it was in the CTR heifers (0.41 to 1.35 mmol/L; SEM = 0.24). On d 22, Streptococcus bovis and Selenomonas ruminantium titers increased after the wheat application, but Megasphaera elsdenii titers increased ($P < 0.05$) only in the FL heifers. In the second experiment, the effect of Bioflavex on the performance and rumen fermentation in finishing heifers was evaluated. Forty-eight Fleckvieh heifers (initial BW = 317 kg; SEM = 5.34) were used in a completely randomized design. Heifers were assigned to 1 of 4 blocks based on their BW and, within each block, assigned to 1 of 2 pens (6 heifers/pen). In addition, 16 heifers (2/pen) were rumen cannulated. Individual BW and group consumption of concentrate and straw were recorded weekly until the animals reached the target slaughter weight. Supplementation with FL did not affect ADG, feed consumption, or feed conversion ratio. Rumen pH and molar proportions of propionate were greater ($P < 0.01$) and acetate proportion was less in the FL ($P < 0.01$) than they were in the CTR heifers. Flavonoid supplementation might be effective in improving rumen fermentation and reducing the incidence of rumen acidosis. This effect of flavonoids may be partially explained by increasing the numbers of lactate-consuming microorganisms (e.g., M. elsdenii) in the rumen.

Key words: acidosis, cattle, flavonoids, high-concentrate diets, rumen fermentation, rumen microorganisms

INTRODUCTION

High-concentrate diets can cause rumen fermentation dysfunctions such as rumen acidosis or bloat (Beauchemin and Buchanan-Smith, 1990). The inclusion of antibiotics (e.g., monensin) in the diet appears to reduce the incidence of those rumen dysfunctions; however, antibiotics as feed additives were banned by the European Community (European Communities, 2003) and flavonoids have been proposed as alternatives to antibiotic therapies (Rhodes, 1996; Broudiscou and Lassalas, 2000). Flavonoids are benzo-1-pyrene derivatives, which are common in fruits, vegetables, nuts, and seeds and have been the subject of medical research (Middleton Jr., et al., 2000) because they have...
anti-inflammatory, antioxidant, and antimicrobial properties (Harborne and Williams, 2000). The effects of flavonoids on rumen fermentation have been the subject of in vitro (Broudiscou and Lassalas, 2000; Yaghoubi et al., 2007) and in vivo experiments (De Freitas et al., 2007). When mixtures of plants flavonoids were tested in a continuous rumen culture system, the flavonoids modified fermentation conditions (pH, propionate proportion, and protein degradation) although the results were not homogeneous (Broudiscou et al., 1999; Broudiscou and Lassalas, 2000).

Bioflavex (FL; Exquim S. A., Barcelona, Spain) is a blend of natural flavonoid extracts comprising mostly naringine (200 g/kg), which is extracted from bitter orange (Citrus aurantium) and grapefruit (Citrus paradisi; 400 g/kg). From the existing literature it was hypothesized that Bioflavex could exert some antimicrobial activities and the objective of this study was to verify the Bioflavex effects on ruminal pH and specific ruminal bacteria involved in lactate production under acidic conditions. In a second experiment, the study examined the effect of flavonoid supplementation on performance and rumen pH in finishing heifers that were fed high-concentrate diets.

**MATERIALS AND METHODS**

Animals were managed following the principles and guidelines of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) Animal Care Committee (no. 3885)

**Animals, Housing, and Diets**

**Experiment 1.** Eight Holstein-Friesian crossbreed heifers (BW = 451.4 kg; SEM = 14.32 kg) were fitted with rumen cannula (10 cm i.d.; Bar Diamond, Parma, ID) and kept indoors in 13.65 × 3.85 m pens at the IRTA experimental station (Prat de Llobregat, Spain). Animals were fed grass hay (11.2% CP, 62.2% NDF, and 34.4% ADF on a DM basis) at a maintenance level (7.2 kg DM/d). The experiment was conducted using a crossover (2 × 2) design that had 2 treatments and 2 periods (22 d each). On d 21 of the experiment, the animals were fasted and on d 22, at 0800 h, rumen acidosis was induced by applying, manually, 5 kg of ground wheat (11.2% CP, 11.0% NDF, and 3.7% ADF on a DM basis) through the cannula. Half of the animals received FL (300 mg/kg), which was mixed in with the ground wheat, and the other half did not receive FL (Control: CTR). After a 15-d transition period, the same 22-d experiment was repeated.

**Experiment 2.** Forty-eight Fleckvieh heifers, with an initial average BW of 317 kg (SEM = 5.34 kg) were weighed on 2 consecutive d and, based on their BW, were assigned to 1 of 4 blocks (12 heifers/block), and each block was assigned to 1 of 2 pens. Randomly, 1 pen of each block was assigned to the FL treatment. The experimental diets were concentrate without (CTR) or with FL (Bioflavex; 300 mg/kg) and barley straw. Concentrate and barley straw were offered ad libitum. Straw and concentrate DMI were calculated weekly based on the difference between the sum of the amounts of food offered daily (0800 h) and the orts of each week. The ingredients and chemical composition of the concentrate are shown in Table 1. Heifers were housed in partially covered outdoor pens (13.65 × 3.85 m) at the experimental station of IRTA (Prat de Llobregat, Spain).

The first 5 d of the experiment were considered an adaptation period. After that period, the average initial BW was 322 (SEM = 3.3) kg and after another 70 d of the experiment, the average BW was 402 (SEM = 5.3) kg, which was close to the target slaughter weight.

Two heifers from each pen (16 animals) were selected randomly and on d 19 of the experiment were fitted with a permanent rumen plastic cannula (1 cm i.d.; Divasa Farmavic S. A., Vic, Spain) inserted into their dorsal rumen sac.

**Measurements and Sample Collection**

**Experiment 1.** From d 19 to 22 of each experimental period, rumen pH was measured every 22 min using a pH meter (X-Mate Pro MX 300, Mettler-Toledo, Barcelona, Spain) that was capable of recording and storing pH values automatically (for details, see Bach et al., 2007). Samples of rumen contents were collected before (0 h) and 2, 4, 8, and 24 h after heifers were given the ground wheat supplement, which were stored frozen (–20°C) until they were analyzed for lactate, NH3–N, and VFA. A 2-mL aliquot of rumen fluid was acidified using 2 mL of 0.2 M HCl and frozen until the NH3–N analysis. In addition, based on Jouany (1982), 2 mL of rumen liquid were mixed with 1 mL of a solution containing 2 g/L mercuric chloride, 20 mL/L orthophosphoric acid, and 2 g/L 4-methylvaleric acid (internal standard) in distilled water and frozen until the VFA analysis. For the microbial DNA analyses, 50-mL rumen samples were centrifuged at 6500 × g for 15 min at 4°C. The supernatant was discarded and the homogenized pellet was distributed among 0.25 g aliquots that were stored frozen at –80°C until they were analyzed.

**Experiment 2.** Animal BW and the consumption of concentrate and straw were recorded weekly. After d 49 of the experiment, rumen fermentation was characterized once a week at 0800 h, 1000 h, and 1200 h. After weighing the animals at 0800 h, the rumen contents (about 200 mL) from the cannulated animals were collected using a vacuum pump, pH was recorded, and the rumen samples were filtered through 2 layers of cheesecloth. Two subsamples were used to quantify NH3–N and VFA concen-
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Table 1. Ingredients and nutrient composition of the concentrate fed to experimental heifers (Exp. 1)

| Item                          | CTR¹ | FL¹ |
|-------------------------------|------|-----|
| **Ingredients, %**            |      |     |
| Corn                          | 44.0 | 44.0|
| Soybean hulls                 | 18.0 | 18.0|
| Wheat brand                   | 17.0 | 17.0|
| Soybean meal                  | 8.00 | 8.00|
| Corn gluten feed             | 5.00 | 5.00|
| Dehydrated alfalfa           | 3.00 | 3.00|
| Palm oil                      | 2.00 | 2.00|
| Calcium carbonate            | 1.00 | 1.00|
| Salt                          | 0.30 | 0.30|
| Vitamin–mineral premix²       | 0.20 | 0.20|
| FL³                           | 0.00 | 0.03|
| **Nutrient Composition, g/kg DM** |      |     |
| Crude protein                 | 132  | 138 |
| Ether extract                 | 58.0 | 62.0|
| Neutral detergent fiber⁴      | 270  | 256 |
| Ash                           | 68.0 | 63.0|

¹CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex (Bioflavex; Exquim S.A., Barcelona, Spain; 300 mg/kg DM ground wheat).
²Declared composition of the vitamin–mineral premix: vitamin A, 200,000 IU/kg; vitamin D₃, 60,000 IU/kg; vitamin E, 1,300 mg/kg; vitamin B₁₂, 125 mg/kg; vitamin B₂, 550 mg/kg; vitamin B₆, 10 mg/kg; zinc oxide, 10 mg/kg; sodium selenate, 60 mg/kg; cobalt carbonate, 60 mg/kg; nicotinic acid, 2 g/kg; magnesium oxide, 9 g/kg; copper carbonate, 7.35 g/kg; and copper sulfate, 2.5 g/kg.
³Bioflavex composition (g/kg): 200 naringine and 400 Citrus aurantium extract.
⁴Assayed using heat-stable amylase.

trations as described above. Urine spot samples (30 mL) were collected from the cannulated heifers using vulva massage at 1200 h, which were frozen immediately and stored at −20 ºC until they were used to quantify the concentrations of purine derivatives (PD; allantoin and uric acid) and creatinine. The urinary PD:creatinine ratio was used as an index of the duodenal absorption of purine bases (PB) and rumen microbial outflow, under the assumption that PB are appropriate microbial markers (Pérez et al., 1997).

**Chemical and Microbial Analyses**

After DM determination using an oven at 60 ºC (until a constant weight was reached), the samples were ground using a hammer mill fitted with a 1.5-mm pore sieve and analyzed for ash (using a muffle oven at 550 ºC for 4 h), CP, and ether extract, following the Association of Official Analytical Chemists (AOAC, 1990). Dietary NDF and ADF concentrations were measured following the method of Van Soest et al. (1991) using sodium sulfite and α-amylase. To measure rumen NH₃–N concentrations, the samples were centrifuged at 25,000 × g for 20 min and the supernatant was analyzed following Chaney and Marbach (1962). Rumen VFA concentrations were measured based on Jouany (1982) method. L+ plus D-lactate was determined by the colorimetric method proposed by Taylor (1996). Urinary concentrations of allantoin, uric acid, and creatinine were measured using the HPLC method described by Balcells et al. (1992). Total microbial DNA was extracted using the repeated bead beating and column (RBB+C) method (Yu and Morrison, 2004) using bead beating in the presence of high concentrations of SDS, salt, and EDTA, and subsequent DNA purifications were performed using QIAamp columns from the Stool DNA Kit (QIAGEN, Valencia, CA).

Quantitative Real-Time PCR (RT-PCR) was performed using 0.2-mL 96-well plates, IQSYBR Green Supermix, and the MyIQ Real-Time Detection System from BioRad (Hercules, CA). For the bacteria quantification, specific primers for regions of the 16S rRNA gene were used at 0.5 µM final concentration. The PCR amplification cycles and primer sequences are presented in Table 2. Amplicon specificity was assessed using melting curve analyses of the PCR end products by increasing the temperature from 55 ºC to 95 ºC at a rate of 0.5 ºC/30 s. The PCR reactions were performed in triplicate and water was used as a negative control. Relative quantification was calculated as a relative expression normalized to a reference sample using the 2^ΔCt method. Absolute expression was quantified using the plasmids derived from pGEM-T vectors (Invitrogen, Carlsbad, CA), which carried the specific amplicons from *Selenomonas ruminantium* and *Streptococcus bovis* as standards.

**Calculations and Statistical Analyses**

Urinary PD:creatinine ratios were measured in the spot samples that were collected from the cannulated heifers. Absolute values of PD (mmol/d) were calculated assum-
ing that creatinine excretion depends on body mass only (Van Niekerk et al., 1963) and then creatinine excretion rate should be equivalent to 896 mmol/kg BW^{0.75} reported by Martin-Orúe et al. (2000) in growing heifers.

The data were analyzed using a mixed-effects model with time considered as repeated measures (SAS Inst. Inc., Cary, NC). For each of the analyzed variables, pen (error term) nested within the treatment was subjected to a compound symmetry variance–covariance structure. In the first experiment, for rumen parameters (except pH) the model included treatment, period, time after feeding (hours, considered as repeated measures), and treatment – time as fixed factors. To analyze changes in pH the model included treatment, feeding phase (before fasting, during fasting, and after acidosis induction), period, and hour (considered as repeated measure correspond to the average time of 3 consecutive pH measurements, which were taken at 22-min intervals) and treatment × hour as the fixed effects.

In the second experiment, the model used to assess concentrate and straw intake and concentrate conversion rate included treatment, block, and time (week), and the interaction between treatment and time was the fixed effect. All of the animals were weighed at the beginning of the experiment and thereafter once a week until the end of the experiment, and the data were used to calculate the ADG as the slope of the linear regression of BW against time. For the rumen and urine data, the effect of time after feeding (hours) and interactions were considered as fixed effects. In the model, pen (n = 8) was the experimental unit for all of the statistical analyses. Significant differences and tendencies were declared at P < 0.05 and P < 0.10, respectively.

RESULTS

Experiment 1

In the first experiment animals did not exhibit evidence of digestive dysfunction and mean BW was stable throughout the experiment [mean initial and final BW of 457 (SEM = 4.2) and 452 kg (SEM = 7.2), respectively].

Changes in the rumen pH during the 72-h period are shown in Figure 1. The interaction between treatment and feeding phase (i.e., maintenance, fasting, and acidosis induction) on rumen pH was significant (P < 0.01). After the ground wheat was administered, rumen pH decreased, more so in the CTR (5.98; SEM = 0.029) than in the FL heifers (6.29; SEM = 0.031). Mean values of ruminal NH₃–N and VFA concentrations and relative VFA proportions are presented in Table 3. Treatment did not affect total VFA concentration or molar proportions of propionate in the rumen. As expected, and paralleling the changes in rumen pH, total rumen VFA concentrations and molar proportions of propionate increased at 2 and 4 h after ground wheat was administered through a rumen cannula; however, at 8 h, concentrations declined. Compared with the CTR heifers, the FL heifers had a decreased rumen molar proportion of acetate (P < 0.05) and tended to have reduced concentrations of butyrate (P = 0.06) and acetate to propionate ratio (P = 0.09). The posttreatment changes in rumen acetate and butyrate (mol/100 mol) were similar to those that were observed in VFA concentrations.

Rumen lactate concentrations, lactate-producing bacteria (S. bovis), and lactate-consuming bacteria (S. ruminantium and M. elsdenii) titers are presented in Table 4. Data correspond to samples that were taken at time 0 (before acidosis onset) and after acidosis induction (the samples from 4 and 8 h after acidosis induction were pooled together, 50:50). Rumen lactate concentrations increased (P < 0.001) after the wheat supplement was administered, and the increase in rumen lactate concentrations between 0 h and 4 to 8 h after wheat supplementation was numerically less (P = 0.09) in the FL heifers (0.41 to 1.18 mmol/L) than in the CTR heifers (0.41 to 1.54 mmol/L; SEM = 0.12). The corresponding titres of S. bovis and S. ruminantium increased (P < 0.05) in the FL and CTR groups after wheat supplementation, and M. elsdenii titers tended (P = 0.09) to increase in the FL heifers. Heifers supplemented with FL had greater (P < 0.05) M. elsdenii titres than CTR heifers.

Experiment 2

Performance data are presented in Table 5. After 70 d of the experiment, heifers reached the target BW. The BW (402 vs. 401 kg; SEM = 5.3) and the ADG (from d 0 to 70; CV = 9.37%) of the FL and the CTR heifers were similar. Treatment did not affect concentrate intake; however, CTR heifers consumed less straw than the FL heifers (0.83 vs. 0.95 kg/d; SEM = 0.30; P < 0.01). Average daily gain decreased (P < 0.01), group concentrate consumption increased (P = 0.1), and the feed conversion ratio did not change over the course of the experiment.

Average pH (6.42 vs. 6.09; SEM = 0.03) and VFA concentrations (74.8 vs. 65.7 mM; SEM = 1.86) in canulated heifers were greater in the FL and rumen pH decreased (6.59, 6.07, and 6.11; SEM = 0.03) and VFA concentrations increased (65.4, 74.3, and 71.2 mM; SEM = 2.22) at 0, 2, and 4 h after concentrate administration. Ammonia-N concentrations were less in the FL than in the CTR heifers [10.2 vs. 41.5 mg/L (SEM = 2.81), respectively].

Rumen molar proportions of acetate (53.9 vs. 59.4 mol/100 mol; SEM = 0.60) were less (P < 0.01) and proportions of propionate (35.5 vs. 28.1 mol/100 mol; SEM = 0.57) were greater (P < 0.01) in the FL than in the CTR heifers; consequently, the acetate-to-propionate ratio was greater (P < 0.01) in the CTR heifers than in
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Table 3. Measures of rumen fermentation after induced acidosis in 8 cannulated Friesian-Holstein heifers (Exp. 1)

| Item                      | Treatment | SEM | 0     | 2     | 4     | 8     | 24    | SEM | Tr   | H    |
|---------------------------|-----------|-----|-------|-------|-------|-------|-------|-----|------|------|
| NH₃–N, mg/L               | CTR (n = 8) | 114 | 107   | 6.28  | 56.1  | 121   | 164   | 99  | 113  | 7.74 |
|                           | FL (n = 8) | 79  | 74.8  | 2.71  | 48.5  | 75.6  | 87.9  | 94  | 78.2 | 3.54 |
| VFA, mM                   |            |     |       |       |       |       |       |     |      |      |
| Acetate                   |            | 69.2| 67.7  | 3.54  | 76.2  | 71    | 68.8  | 64  | 61.4 | 0.63 |
| Propionate                |            | 19.1| 19.6  | 0.4   | 15.4  | 18.3  | 19.8  | 22.8| 20.5 | 0.59 |
| Butyrate                  |            | 9.36| 8.38  | 0.39  | 5.83  | 7.6   | 7.79  | 8.86| 14.2 | 0.53 |
| Acetate to propionate ratio|           | 3.8 | 3.5   | 0.09  | 5.1   | 3.9   | 3.5   | 2.9 | 3.1  | 0.13 |

1CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex (Bioflavex; Exquim S.A., Barcelona, Spain; 300 mg/kg DM ground wheat).

2Time (hours) after a wheat supplement was administered through a rumen cannula.

3Tr = treatment effect; H = time after a wheat supplement was administered through a rumen cannula.

the FL heifers [2.34 vs. 1.65 (SEM = 0.06), respectively]. Changes in the relative proportions of acetate and propionic after concentrate administration (hours) and trough the experimental period (days of experiment) are presented in Table 6 and Figure 2a and 2b. Urinary PD excretion was less ($P < 0.05$) in the CTR heifers than in the FL heifers [101 vs. 121 mmol/d (SEM = 3.24), respectively] and increased ($P < 0.05$) throughout the experiment.

**DISCUSSION**

Acidosis is a digestive disturbance that can be acute, chronic, or subliminal. In the chronic condition, animals might not appear to be sick, but feed intake and performance can be diminished (Owens et al., 1998). Chronic and acute acidosis are indicated by rumen pH values of 5.6 and 5.2, respectively (Cooper and Klopfenstein, 1996). In Exp. 1, in which a wheat supplement was used to induce acidosis, rumen pH was reduced from 6.70 [SEM = 0.22; which is within the normal range for roughage diets (France and Siddons, 1993)] to 5.22 (SEM = 0.19), and the average amount of time in which pH was <5.5 and <6.0 was 0.5 and 4 h, respectively. The minimum pH values (5.0 to 5.5) registered were within the pH ranges reported in studies that used grain engorgement to induce subacute acidosis in (Bauer et al., 1995; Krehbiel et al., 1995; Goad et al., 1998).

During acute acidosis induction (pH 3.9 to 4.5; Nagaraja et al., 1985), lactate concentrations (mmol/L) may exceeded 50; however, under subacute acidosis conditions lower increases in lactate concentrations has been also reported [e.g., <10 (Burrin and Britton, 1986), 5 (Goad et al., 1998), or virtually no increase at all; e.g., <0.4 (Coe et al., 1999; Bevans et al., 2005)]. Moreover in our case, the lack of synchronization between the lowest pH records (9 to 12 h after wheat supply) and lactate sampling (pooled samples harvested at 4 and 8 h) could mask the negative relationship between pH and lactate. Lactic acid accumulates when the amount of rapidly degradable carbohydrates increases suddenly in the rumen, which stimulates the proliferation of rapidly growing lactic acid-producing bacteria, for example, *S. bovis*, to the point where the growth of lactic-producing microorganisms exceeds the growth rate of lactic acid-using bacteria (Russell and Hino, 1985); consequently, lactic acid accumulates. In our experiment, during the acidosis challenge, *S. bovis* titers increased and the maximum titers coincided with the greatest lactate concentrations; however, it is likely that the simultaneous increase in lactate-consuming bacteria (*S. ruminantium* and *M. elsdenii*) might have mitigated the production of lactic acid. After acidosis has been induced (during an adaptation to a high-concentrate diet), there can be a short-term increase in lactate-consuming bacteria (Goad et al., 1998; Tajima et al., 2001) although it has been demonstrated that most lactate-consuming bacteria cannot tolerate low pH conditions (Russell and Hino, 1985). The activity of flavonoids can have an effect on the microbial growth of pathogenic (Wu et al., 2009) and nonpathogenic (Broudiscou and Lassalas, 2000) bacteria. Moreover, in the human intestine the flavonoids genistein and...
Table 4. Effects of Bioflavex before (0 h) and after onset (4 + 8 h) of induced acidosis on lactate concentrations (mmol/L) and rumen populations of *Selenomonas ruminantium*, *Streptococcus bovis*, and *Megasphaera elsdenii* (Exp. 1)

| Items                              | Treatment1 | Time2 | P-value4 |
|-----------------------------------|------------|-------|----------|
|                                   | CTR (n = 8) | 0     | 4 + 8    | SEM | Tr | H | Tr × H |
| Lactate concentration, mmol/L      | 0.97       | 0.41  | 0.24     | 0.08 | 0.44 | <0.01 | 0.09 |
| Absolute quantification4           |            |       |          |     |     |     |        |
| *S. ruminantium*                   | 53,141     | 39,652| 8,679    | 8,374 | 0.72 | 0.02 | 0.72 |
| *S. bovis*                         | 42,677     | 20,207| 9,654    | 9,974 | 0.39 | 0.02 | 0.31 |
| Relative quantification5           |            |       |          |     |     |     |        |
| *S. bovis*                         | 0.51       | 0.25  | 0.13     | 0.13 | 0.8 | <0.01 | 0.87 |
| *S. ruminantium*                   | 0.51       | 0.36  | 0.08     | 0.08 | 0.82 | 0.01 | 0.83 |
| *M. elsdenii*                      | 1.08       | 1.18  | 0.22     | 0.22 | 0.04 | 0.09 | 0.23 |

1CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex (Bioflavex; Exquim S.A., Barcelona, Spain; 300 mg/kg DM ground wheat).
2Time (h) after a wheat supplement; rumen was sampled before (0 h) and 4 and 8 h after acidosis induction but the last 2 samples were (50:50) pooled.
3Tr = treatment effect; H = hour after feeding effect; Tr × H = treatment by hour postfeeding interaction.
4Measurements units, gene molecules 16S-rRNA/12.5 ng of microbial DNA.
5Measurement units, ΔCt (difference of two threshold cycles).

Daidzein has been demonstrated to be able to modify the predominant microbiota (Schoefer et al., 2002; Clavel et al., 2005). Naringin, the main component of Bioflavex, degrades to aglycone (naringenin) in the rumen of sheep (Gladine et al., 2007) and Winter et al. (1989) demonstrated that rumen microflora can break down the aglycone ring into phenylacetic acid, which is an antimicrobial compound. Furthermore, the specific effects of some flavonoids such as (+)-catequins on *Clostridium cocoides* and *Escherichia coli* (Tzounis et al., 2008), (+)-catequins on *Bifidobacterium* (Gibson et al., 1995), daidzein and genistein on *Faeecalibacterium prausnitzii* (Clavel et al., 2005; Decroos et al., 2005), and naringin on *Ruminococcus albus* (Stack et al., 1983) have been described. In our study, the changes in rumen pH that were induced when the animals received FL might have been the result of the effects of flavonoids on the growth of *M. elsdenii*, directly or through their effects on other rumen microbiota. Any direct effects of flavonoids on the growth of *M. elsdenii* have not been confirmed. In any case, the effect seems to be temporary and the rumen ecosystem adapted to the presence of polyphenol compounds.

The addition of the commercial FL mixture was able to partially buffer rumen acidification although this effect was not consistently supported by recorded values in rumen VFA concentration. Discrepancies between rumen VFA concentrations and pH have been observed in both experiments. In the first experiment, large differences in rumen pH between treatments were observed whereas no differences in rumen VFA concentrations between treatments were observed, and in the second experiment FL heifers had greater VFA concentrations and greater rumen pH compared with CTR heifers. Changes in rumen pH are primarily determined by fermentation products, VFA and lactic acid, derived from carbohydrate fermentation (Owens et al., 1998). However, as Sauvant et al. (1999) described, VFA concentration only explained 32% of the variation of the rumen pH observed. Any change in rumen pH is buffered by feed ingested, saliva, and dietary buffers added, and rumen pH is also influenced by rumen rate of passage. In Exp. 1, the lowest pH values were recorded 9 to 12 h after wheat supply and rumen VFA and lactate concentrations were measured latest at 8 h after wheat supply. The lack of VFA and lactate data when the pH records reached the lowest value, avoid to confirm a direct relationship between VFA or lactate concentration.

Table 5. Measures of performance in Fleckvieh heifers (Exp. 2)

| Item                              | Treatment1 | P-value2 |
|-----------------------------------|------------|----------|
|                                   | CTR (n = 24) | FL (n = 24) | SEM | Tr | W | Tr × W |
| BW                                | 325        | 320      | 3.3 | 0.16 | – | – |
| Final, kg                         | 402        | 401      | 5.3 | 0.42 | – | – |
| ADG, kg/d                         | 1.09       | 1.16     | 0.06 | 0.31 | – | – |
| Concentrate intake, kg DM/d       | 6.60       | 6.60     | 0.17 | 0.63 | 0.01 | 0.86 |
| Straw intake, kg DM/d             | 0.83       | 0.95     | 0.30 | 0.01 | 0.70 | 0.86 |
| Feed efficiency4                  | 7.28       | 6.80     | 0.41 | 0.41 | 0.01 | 0.1 |

1CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex (Bioflavex; Exquim S.A., Barcelona, Spain; 300 mg/kg DM ground wheat).
2Tr = treatment effect; W = week effect; Tr × W = treatment by week effect.
3Time corresponds to BW and feed intake of animal, which were recorded weekly.
4Feed efficiency = feed intake:ADG (kg/kg).
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or both) on rumen pH; however, the numerical less lactate concentration in CTR than in FL heifers at 8 h after wheat supply may indicate that FL supplementation prevented heifers from lactic acidosis. Nagaraja andTitgemeyer (2007) indicated that in subacute acidosis, the reason for pH to decline below 5.6 is accumulation of VFA, which is a combination of overproduction (increased substrate) and possibly decreased absorption. Although lactic acid is produced during subacute acidosis, it does not accumulate because lactate-fermenting bacteria remain active and rapidly metabolize it to VFA. However, as the pH nears 5.0 or below for a sustained period, the growth of lactate-fermenting bacteria is inhibited, and hence lactate begins to accumulate. Therefore, subacute acidosis has

Table 6. Rumen fermentation parameters and urinary excretion of purine derivatives in response to a commercial flavonoid supplement (Bioflavex; Exquim S.A., Barcelona, Spain), time after feeding, or days of the experiment in 16 rumen-cannulated Fleckvieh heifers (Exp. 2)

| Item                      | Treatment1 | Treatment1 | Hours2 | Day of experiment3 | P-value4 |
|---------------------------|------------|------------|--------|--------------------|----------|
|                           | CTR (n = 8)| FL (n = 8) | SEM    | 0      | 2  | 4  | SEM    | 49 | 56 | 63 | 70 | SEM |
| Rumen parameters          |            |            |        |        |    |    |        |    |    |    |    |     |
| pH                        | 6.09       | 6.42       | 0.03   | 0.03   | 6.16 | 6.29 | 6.26 | 6.31 | 0.04 | <0.01| <0.01| 0.02| 0.53| 0.15 |
| NH₃–N, mg/L               | 41.5       | 10.2       | 2.81   | 3.44   | 28.4 | 20.5 | 30.1 | 24.5 | 3.97 | <0.01| 0.31 | 0.32| 0.04| 0.66 |
| VFA, mM                   | 65.6       | 74.8       | 1.86   | 2.22   | 66.4 | 70.7 | 74.6 | 69.3 | 2.60 | <0.01| 0.02 | 0.18| 0.52| 0.23 |
| VFA, mol/100 mol          |            |            |        |        |    |    |        |    |    |    |    |     |
| Acetate                   | 59.4       | 53.9       | 0.60   | 0.73   | 58.0 | 55.9 | 56.6 | 55.9 | 0.83 | <0.01| <0.01| 0.27| <0.01| <0.01 |
| Propionate                | 28.1       | 35.4       | 0.57   | 0.68   | 30.3 | 31.0 | 31.7 | 33.9 | 0.80 | <0.01| 0.02 | 0.01| <0.01| <0.01 |
| Butyrate                  | 7.87       | 7.57       | 0.18   | 0.21   | 7.39 | 7.62 | 7.69 | 8.18 | 0.25 | 0.24 | <0.01| 0.19| 0.07| 0.01 |
| Acetate to propionate ratio| 2.34       | 1.65       | 0.06   | 0.07   | 2.18 | 2.08 | 1.90 | 1.82 | 0.08 | <0.01| 0.47 | 0.02| <0.01| <0.01 |
| Urinary excretion of purine derivatives5 |            |            |        |        |    |    |        |    |    |    |    |     |
| PD, mmol/d                | 101        | 121        | 3.24   |        | 105 | 107 | 113 | 120 | 4.58 | 0.02 | –   | 0.05| 0.41| –    |
| PD/Cret, mol/mol          | 1.20       | 1.40       | 0.03   | 1.20   | 1.30 | 1.30 | 1.30 | 1.30 | 0.05 | 0.04 | –   | 0.33| 0.30| –    |

1Treatment effect: CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex (Bioflavex; 300 mg/kg DM concentrate).
2Hours after concentrate administration.
3Days on which the samples were collected for the characterization of the rumen.
4Tr = treatment effect; H = hour after feeding effect; D = day of experiment effect; Tr × D = treatment by day of experiment interaction; Tr × H = treatment by hour postfeeding interaction.
5Urinary excretion of purine derivatives (PD; allantoin + uric acid) collected in the cannulated heifers by vulva massage at 4 h after feeding and expressed as absolute values (total excretion; mmol/d) or expressed by unit creatinine [Cret; excreted (mol/mol)]. Average Cret excretion (896 mmol/kg BW0.75) was described by Martín-Orúe et al. (2000).

Figure 2. Changes in VFA concentration registered in response to a commercial flavonoid supplement (Bioflavex; Exquim S.A., Barcelona, Spain) in 16 rumen-cannulated Fleckvieh heifers (Exp. 2) in relation to time after concentrate was administered [a) interaction of treatment × time after concentrate was given] and over the course of the experiment [b) interaction of treatment × days of experiment (sampling) for acetate and propionate (mol/100 mol)]. CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex.
the potential to become lactic acidosis if the pH of 5.0 is sustained for a time (Nagaraja and Titgemeyer, 2007). In Exp. 2, heifers supplemented FL consumed more straw than CTR heifers; this may stimulate rumination and saliva production buffering rumen pH, explaining the greater pH observed in FL compared with CTR heifers even if rumen VFA concentration was greater in FL than in CTR heifers. As mentioned previously, correlation between rumen VFA concentration and pH is low, and buffering mechanisms such as saliva have a great impact on rumen pH regulation. Mechanisms whereby flavonoid supplementation may stimulate straw consumption are unknown. So, flavonoids addition seems to be effective in preventing pH reduction through modifying the activity of lactating-consuming bacteria but also may have a direct buffer effect or stimulating straw consumption or both. In both of our experiments, flavonoid supplements increased rumen molar proportions of propionate and reduced the acetate-to-propionate ratio, which suggests that flavonoid supplements might have altered the entire rumen microbiota and stimulated the growth of propionate-producing bacteria. Furthermore, FL supplements reduced rumen NH$_3$–N concentrations and increased urinary PD excretion (an index of the duodenal flow of microbial N). The reduction in rumen ammonia concentrations coupled with a significant increase in the duodenal flow of microbial N suggests an improvement in rumen N use.

Despite all of the improvements in rumen fermentation (pH, molar proportion of propionate, and urine PD excretion), there was no apparent improvement in performance and efficiency. Limited information is available on the effects of mixtures of flavonoids on animal performance. Devant et al. (2007) reported that a plant extract supplement did not improve the feedlot performance of Holstein bulls although the plant extract was a mixture of several tertiary compounds including sarsaparilla, a steroidal saponin.

In our study, the slow ADG and the increased residual variation (CV = 9.37%) might have masked the effects of the flavonoid mixture. The heifers consumed large amounts of a corn-based concentrate; however, the CTR heifers did not exhibit rumen pH values that are indicative of subclinical acidosis (Cooper and Klopfenstein, 1996). It was true that FL heifers consumed more straw than the CTR animals, and the regulatory effect of saliva might have altered the pH; however, the small difference (<2% of total DMI) between the 2 groups suggests that any effect of differences in straw intake were negligible. Allowing adequate time for animals to adapt to a high-concentrate diet, the low degradability of corn starch (Owens et al., 1998) and a proper feeding space ratio (Devant et al., 2007; González et al., 2009) reduce the likelihood of acidosis, which would forestall the expression of any effects of flavonoids on the impaired rumen fermentation.

Also, rumen molar proportions of propionate were enhanced in the heifers that were fed FL. Tissues use propionate more efficiently than they use acetate. In addition to direct oxidation through the citric acid cycle, propionate has the potential to be used in gluconeogenesis. However, it is likely that a more efficient use of propionate has a small effect on animal performance (Schelling, 1984). Furthermore, total rumen molar propionate production and absorption are important factors in assessing the impact of propionate on tissue energetics, but rumen concentrations and molar proportions of propionate only indicate the equilibrium between the production and absorption of the end products rather than its absorption.

In our study, an improvement in the duodenal flow of microbial protein (a protein source that has a good AA balance) was reflected in the urine PD excretion of the FL heifers but did not improve heifers ADG. In beef animals fed a high-concentrate diet, up to 45% of dietary protein was found in total duodenal N flow (Martín-Orúe et al., 1998; Devant et al., 2001); therefore, an improvement in rumen microbial yield might have a small effect on total AA flow and, consequently, on animal growth.

In summary, flavonoid supplementation might be effective in improving rumen fermentation and reducing the incidence of rumen acidosis. This effect of flavonoids may be partially explained by increasing the numbers of lactate-consuming microorganisms (e.g., M. elsdeni) in the rumen. However, in the present study despite the positive effects of flavonoid supplementation on rumen pH no effects on performance were observed.

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