Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets

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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, flavenoids, high-concentrate diets, rumen fermentation, rumen microorganisms

INTRODUCTION

High-concentrate diets can cause rumen fermen- tation dysfunctions such as rumen acidosis or bloat (Beauchemin and Buchanan-Smith, 1990). The inclu- sion 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 alterna- tives to antibiotic therapies (Rhodes, 1996; Broudiscou and Lassalas, 2000). Flavonoids are benzo-l-pyrone 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 prop- erties (Harborne and Williams, 2000). The effects of fla- vonoids 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 con- tinuous rumen culture system, the flavonoids modified fermentation conditions (pH, propionate proportion, and protein degradation) although the results were not ho- mogeneous (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 or- ange (*Citrus aurantium*) and grapefruit (*Citrus paradisi*; 400 g/kg). From the existing literature it was hypoth- esized 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 acidotic 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 experi- mental 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 ex- periment 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, manu- ally, 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 ad- aptation period. After that period, the average initial BW was 322 (SEM = 3.3) kg and after another 70 d of the ex- periment, 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 Farma- vic 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, Barce- lona, 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, NH_3–N, and VFA.

A 2-mL aliquot of rumen fluid was acidified using 2 mL of 0.2 *M* HCl and frozen until the NH_3–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 mer- curic 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 ºC. The supernatant was discarded and the homogenized pellet was distributed among 0.25 g aliquots that were stored frozen at –80ºC 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 weigh- ing 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 sub- samples were used to quantify NH_3–N and VFA concen 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 con- centrations 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 bas- es (**PB**) and rumen microbial outflow, under the assump- tion that PB are appropriate microbial markers (Pérez et al., 1997).

Chemical and Microbial Analyses

After DM determination using an oven at 60° C (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 An- alytical 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 NH_3–N concentrations, the samples were centrifuged at 25,000 × *g* for 20 min and the supernatant was analyzed following Chaney and Marbach (1962). Ru- men 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 subse- quent DNA purifications were performed using QIAamp columns from the Stool DNA Kit (QIAGEN, Valencia, CA).

Quantitative Real-Time PCR (**RT-PCR**) was per- formed 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, spe- cific 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 55ºC to 95ºC at a rate of 0.5ºC/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 $2^{\Delta Ct}$ method. Absolute expression was quantified using the plas- mids derived from pGEM-T vectors (Invitrogen, Carlsbad, CA), which carried the specific amplicons from *Selenomo- nas 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 assuming 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 Martín-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 ef- fect. 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 calcu- late 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 consid- ered as fixed effects. In the model, pen (n = 8) was the experimental unit for all of the statistical analyses. Sig- nificant 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 evi- dence 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 in- duction) 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 NH_3–N and VFA concentrations and relative VFA propor- tions 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 ru- men 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; how- ever, 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 propio- nate 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 bac- teria (*S. bovis*), and lactate-consuming bacteria (*S. rumi- nantium* and *M. elsdenii*) titers are presented in Table 4. Data correspond to samples that were taken at time 0 (be- fore acidosis onset) and after acidosis induction (the sam- ples 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* in- creased ($P < 0.05$) 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 simi- lar. 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 consump- tion 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 m*M*; SEM = 1.86) in can- nulated 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 m*M*; SEM = 2.22) at 0, 2, and 4 h after concentrate adminis- tration. 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 the FL heifers [2.34 vs. 1.65 (SEM = 0.06), respective- ly]. Changes in the relative proportions of acetic 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 perfor- mance 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; Nag- araja et al., 1985), lactate concentrations (mmol/L) may exceeded 50; however, under subacute acidosis condi- tions 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 low- est 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 de- gradable 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 micro- organisms exceeds the growth rate of lactic acid-using bacteria (Russell and Hino, 1985); consequently, lactic acid accumulates. In our experiment, during the acido- sis 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 adap- tation 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 demon- strated that most lactate-consuming bacteria cannot tol- erate 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 nonpatho- genic (Broudiscou and Lassalas, 2000) bacteria. More- over, in the human intestine the flavonoids genistein and 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 Biofla- vex, degrades to aglycone (naringein) 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 antimi- crobial compound. Furthermore, the specific effects of some flavonoids such as (+)-catequine on *Clostridium coccoides* and *Escherichia coli* (Tzounis et al., 2008), (+) catequine on *Bifidobacterium* (Gibson et al., 1995), daiztein and genistein on *Faecalibacterium prausnitzii* (Clavel et al., 2005; Decroos et al., 2005),

and naringein 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 ru- men 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 ru- men 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 treat- ments 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 ru- men 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 re- corded 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 (or both) on rumen pH; however, the numerical less lac- tate concentration in CTR than in FL heifers at 8 h after wheat supply may indicate that FL supplementation pre- vented heifers from lactic acidosis. Nagaraja and Titge- meyer (2007) indicated that in subacute acidosis, the rea- son for pH to decline below 5.6 is accumulation of VFA, which is a combination of overproduction (increased sub-strate) and possibly decreased absorption. Although lactic acid is produced during subacute acidosis, it does not ac- cumulate because lactate-fermenting bacteria remain ac- tive 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 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 be- tween rumen VFA concentration and pH is low, and buff- ering 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 micro- biota and stimulated the growth of propionate-producing bacteria. Furthermore, FL supplements reduced rumen NH_3–N concentrations and increased urinary PD excre- tion (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 fermenta- tion (pH, molar proportion of propionate, and urine PD excretion), there was no apparent improvement in per- formance and efficiency. Limited information is avail- able 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 perfor- mance of Holstein bulls although the plant extract was a mixture of several tertiary compounds including sarsa- ponin, 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 ef- fect of differences in straw intake were negligible. Allow- ing adequate time for animals to adapt to a high-concen- trate 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 aci- dosis, 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 ad- dition to direct oxidation through the citric acid cycle, propionate has the potential to be used in gluconeogen- esis. However, it is likely that a more efficient use of propionate has a small effect on animal performance (Schelling, 1984). Furthermore, total rumen molar pro- pionate production and absorption are important factors in assessing the impact of propionate on tissue energet- ics, 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 improve- ment 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 reduc- ing the incidence of rumen acidosis. This effect of fla- vonoids may be partially explained by increasing the numbers of lactate-consuming microorganisms (e.g., *M. elsdenii*) 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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Table 1. Ingredients and nutrient composition of the concentrate fed to experimental heifers (Exp. 1)

Item	CTR[1]	FL[1]
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 premix[2]	0.20	0.20
FL[3]	0.00	0.03
Nutrient Composition, g/kg DM		
Crude protein	132	138
Ether extract	58.0	62.0
Neutral detergent fiber[4]	270	256
Ash	68.0	63.0

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

[2]Declared composition of the vitamin–mineral premix: vitamin A, 200,000 IU/kg; vitamin D , 60,000 IU/kg; vitamin E, 1,300 mg/kg; vitamin B , 125 mg/kg; vitamin B_2, 550 mg/kg; vitamin B_{12}, 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.

[3]Bioflavex composition (g/kg): 200 naringine and 400 *Citrus aurantium* extract.

[4]Assayed using heat-stable amylase.

Table 2. Specific primers for regions of the 16S rRNA and Real-Time PCR (RT-PCR) amplification cycles for bac- teria quantification used in Exp. 1

Microorganism amplification cycle	*16SrRNA* Primers	Reference	RT-PCR
Megasphaera elsdenii	Forward 5′ GACCGAAACTGCGATGCTAGA 3′	Ouwerkerk et al., 2002	1 × (95ºC for 10 min)
	Reverse 5′ CGCCTCAGCGTCAGTTGTC 3′	Ouwerkerk et al., 2002	45 × (95ºC for 15 s, 57ºC for 10 s, and 72ºC for 1 min
Selenomonas ruminantium	Forward 5′ TGCTAATACCGAATGTTG 3′	Tajima et al., 2001	1 × 95ºC for 10 min)
	Reverse 5′ TCCTGCACTCAAGAAAGA 3′	Tajima et al., 2001	45 × (95ºC for 15 s, 53ºC for 10 s, and 72ºC for 1 min
Streptococcus bovis	Forward 5′ CTAATACCGCATAACAGCAT 3′	Tajima et al., 2001	1 × 95ºC 10 min
	Reverse 5′ AGAAACTTCCTATCTCTAGG 3′	Tajima et al., 2001	45 × (95ºC for 15 s, 57ºC for 10 s, and 72ºC for 01 min)

Table 3. Measures of rumen fermentation after induced acidosis in 8 cannulated Friesian-Holstein heifers (Exp. 1)

	Treatment[1]			Time[2]						*P*-value[3]	
Item	CTR (n = 8)	FL (n = 8)	SEM	0	2	4	8	24	SEM	Tr	H
NH_3–N, mg/L	114	107	6.28	56.1	121	164	99	113	7.74	0.2	<0.01
VFA, m*M*	79	74.8	2.71	48.5	75.6	87.9	94.4	78.2	3.54	0.12	<0.01
VFA, mol/100 mol											
Acetate	69.2	67.7	3.54	76.2	71	68.8	64.7	61.4	0.63	0.04	<0.01
Propionate	19.1	19.6	0.4	15.4	18.3	19.8	22.8	20.5	0.59	0.33	<0.01
Butyrate	9.36	8.38	0.39	5.83	7.6	7.79	8.86	14.2	0.53	0.06	<0.01
Acetate to propionate ratio	3.8	3.5	0.09	5.1	3.9	3.5	2.9	3.1	0.13	0.09	<0.01

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

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

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

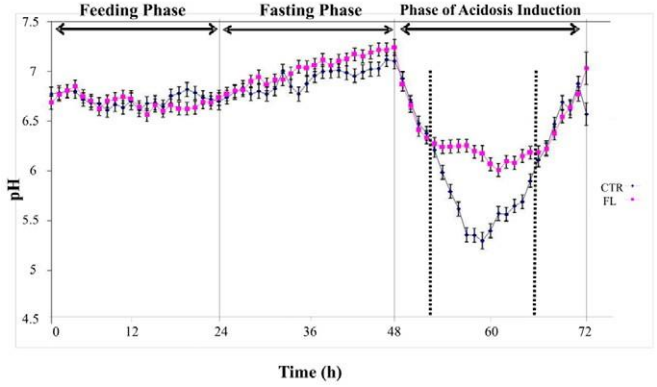

Figure 1. Rumen pH over time (hours) where Holstein crossbreed heif- ers were fed, i) at a maintenance level (feeding phase), ii) 1 d before acidosis was induced (fasting phase, 24 h), and, iii) onset of acidosis induction. Animals were supplied with Bioflavex (FL) or used as a control (CTR), and the dotted lines indicate the time interval in which the differences between 2 treatments were statistically significant ($P < 0.05$; Exp. 1). See online version for figure in color.

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)

	Treatment[1]			Time[2]			*P*-value[4]		
Items	CTR (n = 8)	FL (n = 8)	SEM	0	4 + 8	SEM	Tr	H	Tr × H
Lactate concentration, mmol/L Absolute	0.97	0.80	0.08	0.41	1.35	0.24	0.44	<0.01	0.09
S. ruminantium	53,141	57,499	8,374	39,652	70,988	8,679	0.72	0.02	0.72
S. bovis	42,677	31,183	9,974	20,207	53,564	9,654	0.39	0.02	0.31
Relative quantification[5]									
S. bovis	0.51	0.48	0.13	0.25	0.74	0.13	0.8	<0.01	0.87
S. ruminantium	0.51	0.53	0.08	0.36	0.68	0.08	0.82	0.01	0.83
M. elsdenii	1.08	1.46	0.22	1.18	1.35	0.22	0.04	0.09	0.23

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

[2]Time (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; rumen was sampled before (0 h) and 4 and 8 h after acidosis induction but the last 2 samples were (50:50) pooled.

[3]Tr = treatment effect; H = hour after feeding effect; Tr × H = treatment by hour postfeeding interaction.

[4]Measurements units, gene molecules 16S-rRNA/12.5 ng of microbial DNA.

[5]Measurement units, ΔCt (difference of two threshold cycles).

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

	Treatment[1]			*P*-value[2]		
Item	CTR (n = 24)	FL (n = 24)	SEM	Tr	W[3]	Tr × W
BW						
Initial, kg	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 efficiency[4]	7.28	6.80	0.41	0.41	0.01	0.1

1CTR = control (did not receive Biofl avex); FL = heifers supplemented with Biofl avex (Biofl avex; 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 D_3, 60,000 IU/kg; vitamin E, 1,300 mg/kg; vitamin B_1, 125 mg/kg; vitamin B_2, 550 mg/kg; vitamin B_{12}, 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.

3Biofl avex composition (g/kg): 200 naringine and 400 *Citrus aurantium* extract.

4Assayed using heat-stable amylase.

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)

	Treatment[1]			Hours[2]				Day of experiment[3]					*P*-value[4]				
Item	CTR (n = [illegible])	FL (n = [illegible])	SEM	0	2	4	SEM	49	56	63	70	SEM	Tr	H	D	Tr × D	Tr × H
Rumen parameters																	
pH	6.09	6.42	0.03	6.59	6.07	6.11	0.03	6.16	6.29	6.26	6.31	0.04	<0.01	0.01	0.02	0.53	0.15
NH_3–N, mg/L	41.5	10.2	2.81	27.0	28.9	21.7	3.44	28.4	20.5	30.1	24.5	3.97	<0.01	0.31	0.32	0.04	0.66
VFA, m*M*	65.6	74.8	1.86	65.3	74.2	71.1	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	58.8	53.7	57.4	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	30.4	33.1	31.7	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	7.03	8.55	7.58	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	2.07	1.92	1.99	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 derivatives[5]																	
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	[illegible]	0.05	0.04	–	0.33	0.30	–

[1]Treatment effect: CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex (Bioflavex; 300 mg/kg DM concentrate).

[2]Hours after concentrate administration.

[3]Days on which the samples were collected for the characterization of the rumen.

[4]Tr = 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.

[5]Urinary 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 $BW^{0.75}$) was described by Martín-Orúe et al. (2000).

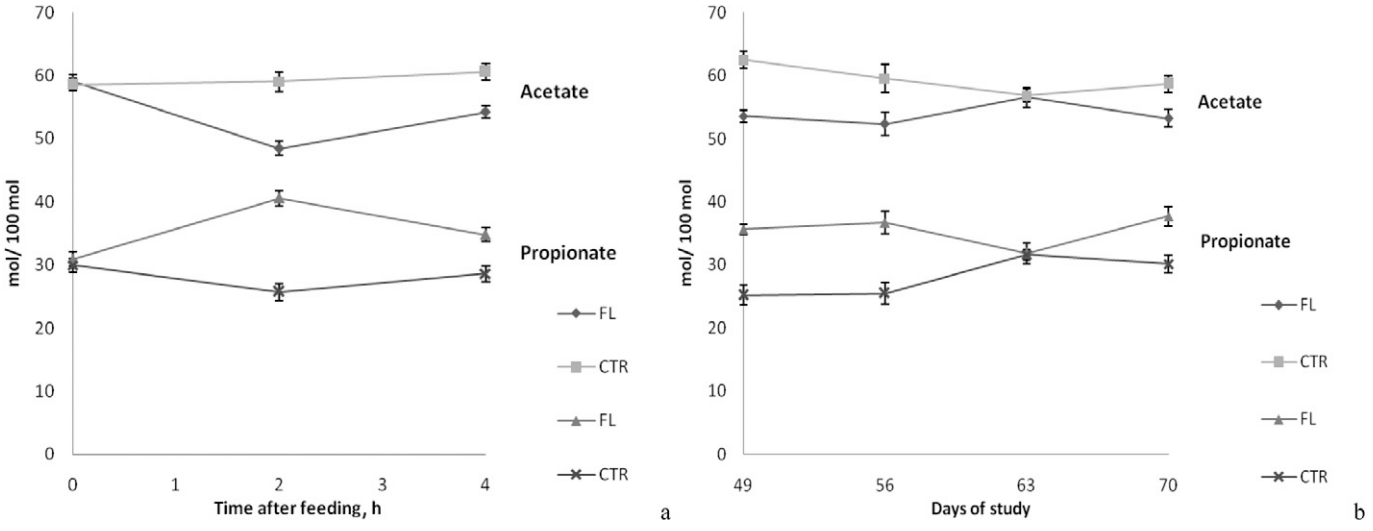

Figure 2. Changes in VFA concentration registered in response to a commercial flavonoid supplement (Bioflavex; Exquim S.A., Barcelona, Spain) in 16 rumen-cannulated Fleckvieh heifers (Exp. 2) in relation to time after concentrate was administered [a) interaction of treatment × time after concentrate 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.