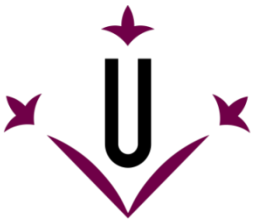

Effects of the citrus flavonoid extract Bioflavex or its pure components on rumen fermentation of intensively reared beef steers

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Abstract.

Two experiments were performed to study the effects of the citrus flavonoid extract Bioflavex (BF; Interquim SA, FerrerHealthTech, Sant Cugat, Barcelona, Spain) or its components on the rumen fermentation of a high-concentrate diet. In an *in vivo* experiment, eight Friesian steers (398 ± 12.2 kg bodyweight) fitted with a rumen cannula were given a basal concentrate (CTR) or a CTR supplemented with BF (450 mg/kg dry matter, DM) in a 2 · 4 crossover design. No differences were observed in performance parameters of BF and CTR steers. Diet BF increased pH values and the molar proportion of propionate and reduced lactate concentration as a result of an increase in the relative abundance of lactate-consuming microorganism *Selenomomas ruminantium* ($P < 0.01$) and *Megaesphaera elsdenii* ($P = 0.06$). In an *in vitro* experiment, the effect of BF and its pure flavonoid components added to the incubation medium was studied separately. Bioflavex and its main components naringine, neohesperidine (NH) and poncirine (PC) were added to the incubation medium at 500 mg/g DM, with the unsupplemented substrate also included as a control (CTR). After 12 h of incubation, flavonoid mixture and NH and PC reduced ($P < 0.01$) the volume of gas produced and the molar proportion of acetate ($P < 0.01$), and increased that of propionate ($P < 0.01$). PC reduced the relative quantification of *Streptococcus bovis*, whereas NH and BF increased the relative quantification of *M. elsdenii* in relation to CTR ($P < 0.01$). Bioflavex supplementation in steers in feedlot was effective in preventing a collapse in pH and it enhanced rumen fermentation efficiency through modifying the activity of lactate-consuming bacteria and a greater molar proportion of propionate and a reduction of that of acetate, suggesting its positive role in modulating the activity of rumen microbiota.

Additional keywords: Holstein steers, intensive beef production, *in vivo*, *in vitro*, plant secondary metabolites.

Introduction

Antibiotic therapies formerly used to alleviate dysfunctions of rumen fermentation (i.e. acidosis or bloat) when high concentrate rations are given to improve rumen efficiency and animal performances (Beauchemin and Buchanan-Smith 1990) are banned in Europe (European Communities 2003) because of increasing awareness of hazards associated with antibiotics, i.e. presence of chemical residues in animal-derived foods and development of bacterial resistance to antibiotics together with consumer demands on food quality and safety. Removal of antibiotics forced nutritionists to explore some natural less aggressive substances as alternatives (Patra and Saxena 2009) and secondary plant compounds such as flavonoids have been proposed as an alternative to antibiotic therapies (Rhodes 1996). Balcells *et al*. (2012) showed previously that addition of Bioflavex (BF; Interquim SA, Barcelona, Spain), a blend of natural flavonoids extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*), was effective in preventing decline of ruminal pH in heifers subjected to experimentally induced acidosis. It also increased rumen molar proportions of propionate and prevented lactate accumulation by creating conditions that favoured lactate-consuming microorganisms such as *Megasphaera elsdenii*. The assay was designed (1) to confirm the *in vivo* effect of flavonoid citrus extract (Bioflavex) used at a commercial doses (450–500 mg/kg dry matter, DM), using rumen fistulated steers fed high concentrate rations, and (2) to discriminate the individual effect of each flavonoid component of the commercial flavonoid mixture (BF) under *in vitro* conditions.

Materials and methods

In vivo *study*

The present experiment was conducted in the facilities of the Servicio de Experimentación Animal of the University of Zaragoza (Spain). All animal care, handling and surgical procedures were approved by the Ethics Committee of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD 1201/05, which meets the Directive 2010/63/ EU on the protection of animals used for experimental and other scientific purposes.

Eight 9-month-old Holstein-Friesian group-housed steers (398 ± 12.2 kg) were fitted with a cannula (88 mm length and 10 mm i.d., DIVASA Farmavic SA, Vic, Barcelona, Spain) in the dorsal sac of the rumen. After recovery from cannulation, animals were housed in 3.2 · 1.7 m individual pens with concrete floor, and were provided with an automatic water dispenser and separate concentrate and forage feeders. Animals were randomly assigned to each of two experimental treatments, based on the offer of a standard concentrate mixture plus barley straw (CTR; Table 1), or on the concentrate supplemented (450 g/kg) with the commercial mixture of flavonoid BF, comprising mostly naringine (NG), which is extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*), also including neohesperidine (NH) and poncirine (PC) plus tracer amounts of hesperidine(HS), isonaringine (IN) and neoeriocitrine (NE).

The experiment lasted for 24 days and was organised in a 2 · 4 crossover design, with two periods of 12 days and four steers per period and treatment. Concentrate and straw were supplied *ad libitum* in separated feeders, the former in only one dose (at 0800 hours) and the latter in three daily doses, allowing for an *ad libitum* availability, and consumptions and orts were recorded daily on a DM basis. Steers were weighed individually when starting and at the end of each experimental period. The first 10 days from each period were designated for adaption of the rumen environment to the treatments (adaptation period), whereas in the following 2 days, an approximate volume of 100 mL was sampled from the rumen with the help of a vacuum pump (automatic vacuum device Fazzini F-36.00, Rome, Italy), at 0, 4 and 8 h after the concentrate supply. Rumen pH was immediately recorded using a pH meter (Model 507, CRISON Instruments SA, Barcelona, Spain). Then, rumen contents were filtered through a 1-mm pore-size metal mesh, and 0.3 mL were transferred to an eppendorf tube, weighed and immediately frozen in liquid nitrogen and stored at -80^{o}C until further molecular analysis. Other three 4 mL subsamples were pipetted into empty tubes or tubes containing either 1 mL of a 0.34 M orthophosphoric acid and 0.016 M of 4-methylvaleric acid solution or 4 mL of 0.2 N HCl, that were stored at -20^{o}C until further analysis of lactate, volatile fatty acids (VFAs) and ammonia concentration respectively.

In vitro *study*

The effect of BF mixture or its pure flavonoid components (NG, NH, PC, HS, IN and NE) on rumen fermentation were tested against the control (CTR) at a dose of 500 mg/g DM of the incubated substrate, in an *in vitro* incubation system (Theodorou *et al*. 1994). Four batches or incubation series were conducted using rumen liquor from four rumen-cannulated growing steers, using a different one as donor of inoculum on each incubation series. Animals were fed a concentrate (BF-free) ration consisting of a proportion of 0.90 of a commercial concentrate and 0.10 of barley straw (Table 1) offered *ad libitum* for at least 4 weeks. The rumen contents were sampled at 0800 hours and filtered through a double layer of gauze and used immediately as an inoculum at 10% of total incubation volume. In each incubation series, four serum glass bottles (120 mL) per each experimental treatment were filled with 80 mL of an incubation solution prepared under a CO_2 stream (Mould *et al*. 2005), including rumen inoculum, mineral and buffer solutions plus a reducing solution made up with cysteine hydrochloride. A mixture of 600 mg of the same concentrate given to steers plus 60 mg of barley straw was used as the substrate. The concentrate and the barley straw were milled through a 1-mm sieve. Flavonoids were added to the incubation medium, and the substrate without flavonoids was considered as a control (CTR). Bottles were sealed with butyl rubber stopper and aluminum crimps and incubated at 39 ± 1^{o}C in a shaking water bath for 12 h.

Pressure measurements were determined with a TP704 incubation temperature. Gas volume at each incubation time was expressed per unit of DM incubated substrate. At the onset (Time 0) of each incubation series, two samples were taken from the prepared incubation solution (stock solution). At 12 h post incubation, bottles were opened, their pH determined (pH-meter 2000, CRISON Instruments, Barcelona, Spain) and 12 mL of the incubation media was weighed, immediately frozen in liquid nitrogen and stored at -80^{o}C for microbiota analyses. The remaining content was filtered through a metal sieve (1-mm mesh size) and sampled for subsequent analyses of lactate, ammonia nitrogen and VFA concentration, as above. Samples

were immediately frozen (-20^{O}C) until further analyses.

Microbial and chemical analyses

All the samples taken from the *in vivo* and *in vitro* experiments underwent DNA extraction using a QIAamp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK), following the manufacturer's instructions. Relative abundance of *S. bovis*, *S. ruminantium* and *M. elsdenii* in relation to the total bacteria were determined using specific primers. The relative quantification was conducted using the 2^{-DCt} method (Livak and Schmittgen 2001). Analyses were performed on CFX96 Touch real-time polymerase chain reaction (PCR) detection system (BioRad, Laboratories Inc., Hercules, CA, USA). PCR amplification conditions were as follows: 1 cycle of 95^{O}C for 10 min, and 40 cycles of 95^{O}C for 15 s, 60^{O}C for 10 s and 72^{O}C for 55 s; amplicon specificity was assessed using melting curve analyses of the PCR end products by increasing the temperature from 55^{O}C to 95^{O}C at a rate of 0.5^{O}C/30 s. The primer sets and amplicon sizes are described in Table 2.

The DM content of the concentrate and straw was determined after drying at 105^{O}C until a constant sample weight. Ash content was determined by incineration on muffle furnace (550^{O}C for 4 h) and crude protein was analysed by the Kjeldahl method (ref. 976.05) according to AOAC (1990). Neutral detergent fibre was determined according to Van Soest *et al.* (1991) procedures, using a amylase, and discounting ashes from the residue. Ammonia concentration was determined by the Chaney and Marbach (1962) method after sample centrifugation (2500*g*, 20 min). The VFA concentrations were determined by gas chromatography, on the basis of the technique of Jouany (1982) using a capillary column (BP21 30 m · 0.25 mm i.d. · 0.25 mm, SGE Analytical Science, Australia). Lactate was measured using the method of Taylor (1996).

Statistical analyses

In vivo rumen-fermentation parameters (pH, lactate, ammonia and VFA concentration and proportions of bacterial species) were analysed in a completely randomised design with a crossover arrangement, using four animals in each group (control vs flavonoid), by ANOVA using the mixed model of SAS (SAS Institute Inc., Cary, NC, USA). The model included period, treatment, sampling day and time post-feeding (hours, considered as repeated measures) and treatment · time as fixed factors, animal nested within the period was considered as a random factor and experimental unit. Concentrate and straw intake, concentrate conversion rate and average daily gain were analysed by ANOVA using the general linear model of SAS. The model included treatment and period, and their interaction was the fixed effect.

Results from the *in vitro* trial were studied using a completely randomised block design, including four different blocks. Four different animals with same basal diets (concentrate plus barley straw) and no flavonoids in their diets were used to collect rumen fluid in different days and the incubation was repeated for four times in four different days. Each animal donor, which agrees with the incubation series, was considered as the experimental unit. Computations for gas-production values were performed by

ANOVA using the mixed model of SAS, each experimental series was considered as a random effect. The model included block, treatment, time (considered as repeated measures) and treatment · time as fixed factors. Fermentation parameters were analysed using the general linear model, including block and treatment as fixed factors.

For both experiments, the Tukey multiple comparison procedure was applied to all treatments and significant differences and tendencies to differences were declared at $P < 0.05$ and $0.05 < P < 0.10$ respectively.

Results

In vivo *study*

There were no differences between CTR and BF in daily concentrate (7.42 vs 7.79 kg DM/day, $P = 0.36$) or straw intake (0.83 vs 1.05 kg DM/day, $P = 0.33$), nor in bodyweight gain (1.4 vs 1.4 kg/day, $P = 0.99$) along the experimental period.
The average pH values for steers given BF were higher ($P < 0.01$) than those for steers that did not receive BF (CTR group) in their basal concentrate (Table 3) and they dropped significantly ($P < 0.01$) 4 h after feeding. No interaction between treatment and time post-feeding was detected. Ammonia concentration in CTR steers was higher ($P < 0.01$) than in BF ones, and, in both groups, it was reduced after feeding ($P < 0.01$). Total VFA concentration was not altered by the flavonoid supplementation, but it increased from 0 to 4 h after feeding ($P < 0.01$) in both treatments. Despite the increased molar proportion of propionate with flavonoid addition ($P = 0.05$), it occurred only at 0 h, whereas no treatment differences were recorded at 4 and 8 h (interaction treatment · hour; $P < 0.01$). The opposite trend occurred with acetate proportion, which was lower at 0 h with BF than CTR but did not differ afterwards (interaction treatment · hour; $P < 0.01$).

No treatment differences were recorded for molar proportions of the other VFAs, except that of butyrate increasing, and those of isobutyrate and isovalerate decreasing, from 0 to 4 h (time effect, $P < 0.01$). Lactate concentration was reduced in BF compared with CTR ($P < 0.01$), and, for both treatments, it increased from 0 to 4 h after feeding ($P < 0.01$). Relative abundance of *S. bovis* was not affected by treatment (Table 4), but it decreased linearly with time ($P = 0.016$) in both treatments. In contrast, the average abundance of *S. ruminantium* increased ($P < 0.01$) with BF supplementation, whereas *M. elsdenii* had also a tendency to increase ($P = 0.06$). The relative abundance of *M. elsdenii* increased with time ($P = 0.02$) in both treatments, whereas the relative abundance of *S. ruminantium* remained unaffected from 0 to 4 h post-feeding, but increased at 8 h in BF, whereas it tended to decrease in CTR animals (3.74 and 1.99 respectively, interaction treatment · time; $P = 0.054$).

In vitro *study*

Addition of BF to the culture media reduced gas production ($P < 0.01$; Table 5) in relation to the CTR after 12 h of incubation, as was also the case for its main components NH and PC ($P < 0.05$). Although also NG numerically reduced gas production, this reduction was not statistically significance. Tracer components did not alter gas production in relation to values recorded with CTR bottles.

The pH values were maintained between 6.7 and 6.8 during the 12-h incubation period and no treatment differences were observed ($P > 0.10$, data not shown). Addition of flavonoid substances had a small effect on ammonia concentration, where a reduction ($P = 0.03$) was detected only in bottles supplemented with IN (Table 6). Treatments did not significantly ($P > 0.10$) affect total VFA concentration or molar proportions of butyrate and minor VFAs. Supplementations of BF and its individual compounds all increased ($P < 0.01$) the molar proportion of propionate, but reduced ($P < 0.01$) that of acetate.

No significant differences were observed in lactate concentrations after 12 h of incubation with the addition of flavonoid substances, compared with CTR (Table 7). Relative quantification of the lactate-producing *S. bovis* showed a decrease ($P < 0.01$) with NH, PC and IN, and an increase with NE, compared with CTR, whereas the proportion of the lactate-consuming bacteria *M. elsdenii* increased with BF and NH ($P = 0.01$) and *S. ruminantium* increased with HS ($P = 0.01$).

Discussion

Rearing beef cattle by using high-concentrate diets may lead to a rumen pH reduction and may induce subacute (pH 5.6–6.0; Goad *et al*. 1998) or acute (pH below 5.6; Nagaraja *et al*. 1985) acidosis. Rumen acidosis is caused by both a reduction in salivation, due to shortage of fibre size, and an increase in VFA production and in lactic acid accumulation. Moreover, starch availability increases the presence of glucose-utilising bacteria through the lactate pathway (such as *S. bovis*) and the higher lactate concentration may consequently increase, to a certain extent, the relative abundance of lactic-consuming bacteria such as *M. elsdenii* (Goad *et al*. 1998). During induced experimental acidosis by introducing a high grain supply through the rumen cannula, an addition of 300 mg BF/kg DM was effective in preventing pH reduction (Balcells *et al*. 2012); however, in a simultaneous trial using growing heifers, the positive effect of the citrus flavonoid blend was less conclusive at the rumen metabolism level and no improvement in average daily gain was detected.

Steers consumed, on average, 0.89 kg/kg of their diet as concentrate, and concentrate was consumed mostly after offering; consequently, rumen pH values dropped reaching subacute values from 4 to 8 h after feeding. Allowing the animals to adapt to the high-grain diets and the low degradability of corn starch might reduce the possibility of acute acidosis (Owens *et al*. 1998). Average pH and its daily evolution in CTR steers agreed well with previous reports under similar experimental conditions (Goad *et al*. 1998; Beauchemin *et al*. 2001; Koenig *et al*. 2003). Confirming our previous work (Balcells

et al. 2012), BF addition increased average pH in 0.3 pH units, and BF-fed steers tended to have a faster recovery of normal pH values after concentrate feeding than did those given CTR.

It is expected that the effect of BF on microbial environment of the rumen should relay on the activity of its main flavonoid components of the mixture (i.e. NG, NH and PC). Indeed, NG and NH reduced the volume of gas produced at a similar level, as an index of microbial activity. Moreover, the relationship between the activity and dosage of flavonoids seems to indicate the existence of a threshold level, considering that all pure compounds were added to the same level, and that the observed reduction in gas production was equivalent in magnitude. Previous results from our group under similar experimental conditions using a lower dose (Seradj *et al*. 2014) did not demonstrate any positive relationship between flavonoids and methane production.

Anti-bacterial activity of flavonoids (Broudiscou and Lassalas 2000; Wu *et al*. 2009) has been reported through the cleavage of the flavonoid C-ring to produce the toxic phenolic acids (Schoefer *et al*. 2002). Degradation of NG (as the main component of BF) similar to that of the corresponding aglycone (Gladine *et al*. 2007), and further C-ring cleavage to produce anti-microbial compounds may also explain the anti-microbial activity detected by NG (Winter *et al*. 1989). Although it is true that the improvement in pH values and the significant reduction in gas production may reflect a reduction in rumen microbial fermentation, such anti-microbial activity must have some specificity, given that relative abundance of lactate-consuming microorganism was not reduced, but, rather, increased. Authors are not aware of any anti-microbial mechanisms of action for NH, but probably the similarity between both NH and NG structures may suggest a common metabolic pathway.

Rumen ammonia concentration reflects the balance between production (mostly from protein degradation) and the summation of microbial utilisation and absorption through the rumen epithelium, which does not exist *in vitro*. Previous papers have shown the activity of flavonoids against protein degradation (Broudiscou *et al*. 2002) or increasing duodenal nitrogen flow, which suggest an increase in microbial nitrogen usage (Balcells *et al*. 2012). However, the fact that differences in ammonia concentration have a minor relevance *in vitro* suggests that the differences observed *in vivo* may be explained by differences in absorption mechanism. Bioflavex was able to buffer rumen pH, and this seems to be clearly related with the drop in lactate concentration with BF, although this effect was not consistently supported by the values of rumen VFA concentration *in vivo*. Rumen pH values are primarily determined by fermentation end products (i.e. VFA, lactate, ammonia; Owens *et al*. 1998), although VFA explains only ~32% of the total observed variation in rumen pH (Sauvant *et al*. 1999) and changes in feed and water intake, saliva production, dietary buffers and rate of passage may contribute to explain this effect. In both *in vivo* and *in vitro* trials, BF supplementation consistently increased rumen molar proportion of propionate at the expense of acetate, and the effect of activity of BF and its components on molar VFA profiles was confirmed in the *in vitro* trial, where the absorption process is excluded. Tissues use propionate more efficiently than they do acetate; in addition, propionate may enter the citric acid cycle to produce oxaloacetate, which can then be used to produce glucose via gluconeogenesis. However, production of VFA was not reflected on productive parameters, in agreement with the results of Schelling (1984) for animals fed high-concentrate diets.

These changes observed in the VFA fermentation profile suggest that flavonoids might have modified the digestive microbiota. This has been confirmed in the human gut, where genistein is able to modify prevalent species of microbiota (Schoefer *et al*. 2002; Clavel *et al*. 2005). The specific effect of flavonoids has also been demonstrated in the rumen ecosystem, such as that of catechin on *Escherichia coli* (Tzounis *et al*. 2008) and *Bifidobacterium* (Gibson *et al*. 1995), NG on *Ruminococcus albus* (Stack *et al*. 1983) and both NG and NH against archaeas such as hydrogenotrophic methanogens and *Methanosarcina* ssp. (Seradj *et al*. 2014). Balcells *et al*. (2012) showed that the induced differences caused by Bioflavex supplementation during the days of study (49–70 days) were gradually diminished, and concluded that rumen environment in growing heifers could be adapted in a short term, thus diminishing the impact of the presence of Bioflavex, which suggests that the optimum effect of the citrus flavonoid blend could rather be expected in short administration periods, such as here. In our study, the inclusion of BF in diet did not alter the relative abundance of lactate-producing bacteria *S. bovis*, but tended to increase that of the lactate-consuming species *M. elsdenii* and *S. ruminantium*, although no changes in the abundance of *S. ruminantium* were previously detected (Balcells *et al*. 2012). In the latter work, acidosis was suddenly induced by supplying 5 kg of ground wheat through the rumen cannula, and rumen pH was progressively recovered, the effects of such acidosis induction on rumen environment, might explain such discrepancies.

The *in vitro* trial supported the results of the *in vivo* trian, where the addition of BF increased the abundance of both *M. elsdenii* and *S. ruminantium* ($P < 0.01$), without any effect on *S. bovis*. However, the *in vitro* effect of BF flavonoid components was not homogeneous on *S. bovis*, and NH reduced and NE increased the abundance of *S. bovis*, whereas NG did not affect it. In contrast, the effect of flavonoids on *M. elsdenii* was consistent, with BF and its main components increasing its concentration *in vitro*.

Owens *et al*. (1998) observed a negative relationship between rumen pH and lactate concentration in the *in vivo* trial, with pH decreasing after concentrate feeding due to lactate accumulation. However, only numerical differences in lactate concentration were detected in the CTR bottles when the effect of BF and its components were tested *in vitro*, suggesting that lactate absorption under conditions of subacute acidosis may play a crucial role in lactate accumulation *in vivo*. Nevertheless, a different behaviour in lactate metabolism between the *in vivo* and *in vitro* could not be discarded. Lactate accumulation during acidosis induction may vary largely, exceeding 50 mmol/L in acute acidosis (Nagaraja *et al*. 1985), although values lower than 10 mmol/L have been described during conditions of subacute acidosis (Burrin and Britton 1986; Goad *et al*. 1998), and there have been even cases in which concentration was unaffected (Bevans *et al*. 2005). In our experiment, when subacute pH conditions were reached after feeding, a significant lactate accumulation was detected. Moreover, during acidosis challenge, *S. bovis* titters increased, with the maximum occurring together with the greater lactate concentration. It is likely that the significant increase in lactate-consuming bacteria promoted by BF might have reduced the lactic acid accumulation as it should be a mechanism able to justify the mitigation of rumen acidity induced in BF fed animals. The enhancement of lactate-consuming bacteria with BF was confirmed *in vitro*, where a positive effect of BF and its main components on the relative abundance of lactate-consuming bacteria was evidenced.

In summary, BF supplementation in high concentrate-fed steers was effective in preventing the pH reduction and enhancing rumen fermentation efficiency through modifying the activity of lactate-consuming bacteria and modulating rumen fermentation towards a higher molar proportion of propionate and reducing that of acetate, which may suggest that the supplementation with flavonoids did have a role in activity of rumen microbiota.

Acknowledgements

This research was financed by Interquim SA (FerrerHealthTech), Sant Cugat, Barcelona. A. R. Seradj was a recipient of a grant from the Government of Catalonia (FI-DGR 2011) and A. Gimeno received a FPI grant from the MICINN (Spanish Government).

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Table 1. Ingredients (g/kg) of the basal concentrate and chemical composition (g/kg DM) of the concentrate and straw used

The basal concentrate supplemented with the commercial mixture of flavonoids (Bioflavex, BF) at 450 mg/kg DM of the concentrate for the BF treatment

Parameter	Basal concentrate (g/kg)	Barley straw
Ingredient		
Corn grain	350	
Barley grain	250	
Soybean meal (44%)	100	
Wheat bran	60	
Sunflower meal (30%)	35	
Gluten feed (20%)	80	
Beet Pulp	70	
Palm oil	25	
Calcium	13	
Bi-calcium phosphate	8	
Salt	3	
Mineral and vitamin premix[A]	4	
Sepiolite	2	
Chemical composition		
Dry matter (g/kg DM)	906	890
Organic matter (g/kg DM)	931	933
Crude protein (g/kg DM)	154	38
Neutral detergent fibre (g/kg DM)	292	744

[A]Mineral and vitamin premix (IU/kg): vitamin A, 5000; vitamin D3, 800; (mg/kg): vitamin E, 12; Zn (from zinc oxide), 80; Se (from sodium selenate), 0.15; Co (from cobalt carbonate), 0.2; Mg (from magnesium oxide), 32; Cu (from copper sulfate), 3.18; Fe (from ferrous carbonate), 24; K (from potassium iodide), 0.4.

Table 2. Specific primer sets for quantitative polymerase chain reaction (qPCR) used in the both experiments
qPCR conditions: 1 · (958C 10 : 00 min), 40 · (958C 15 s, 608C 10 s, 728C 55 s) + melt curve

Target	Sequences	Reference	Amplicon size (bp)
Total bacteria	F 5′-GTGSTGCAYGGYTGTCGTCA-3′ R 5′-ACGTCRTCCMCACCTTCCCC-3′	(Maeda *et al.* 2003)	120
Selenomomas ruminantium	F 5′-TGCTAATACCGAATGTTG-3′ R 5′-TCCTGCACTCAAGAAAGA-3′	(Tajima *et al.* 2001)	513
Streptococcus bovis	F 5′-CTAATACCGCATAACAGCAT-3′ R 5′-AGAAACTTCCTATCTCTAGG-3′	(Tajima *et al.* 2001)	869
Megasphaera elsdenii	F 5′-GACCGAAACTGCGATGCTAGA-3′ R 5′-CGCCTCAGCGTCAGTTGTC-3′	(Ouwerkerk *et al.* 2002)	130

Table 3. Pattern of rumen fermentation parameters in intensively fed growing steers given concentrate with (BF) or without (CTR) the flavonoid mixture (Bioflavex)

T, treatment effect; H, sampling time; T × H, interaction of the treatment and time post-feeding. VFA, volatile fatty acid. Values in the Mean column within a parameter followed by different letters are significantly different among the sampling times (at $P = 0.05$). Values within a row followed by different lower-case letters are significantly different between the treatments (at $P = 0.05$). Different upper-case letters within a row indicate a significant interaction effect (at $P = 0.05$)

Parameter	Time (h)	Treatment			s.e.m.	*P*-value		
		CTR	BF	Mean		T	H	T × H
pH	0	6.8	7.0	6.9j	0.09	0.002	0.001	0.374
	4	5.4	5.5	5.4k				
	8	5.3	5.7	5.5k				
	Mean	5.8b	6.1a					
NH_3 (mg/L)	0	51.2a	28.9b	40.0j	4.67	0.001	0.001	0.172
	4	10.8	5.9	8.40k				
	8	21.9	9.4	15.7k				
	Mean	28.0a	14.7b					
Lactate (mM)	0	1.04	0.6	0.81j	1.74	0.001	0.001	0.221
	4	2.41	1.01	1.71k				
	8	2.75	1.11	1.83k				
	Mean	2.08a	0.91b					
VFA (mM)	0	53.3	67.9	60.60k	6.40	0.407	0.001	0.371
	4	113.2	114.5	113.8j				
	8	109.9	107.5	108.7j				
	Mean	92.1	96.7					
Acetate	0	67.2X	61.8Y	64.5j	1.14	0.19	0.001	0.008
	4	59.7	60.5	60.1k				
	8	58.9	59.6	59.3k				
	Mean	61.9	60.6					
Propionate	0	17.9Y	24.2X	21.0k	1.01	0.054	0.001	0.001
	4	24.7	24.2	24.4j				
	8	25.1	24.3	24.7j				
	Mean	22.5b	24.2a					
Butyrate	0	9.4	8.7	9.0k	0.73	0.439	0.001	0.922
	4	11.2	10.7	10.9j				
	8	11.5	11.3	11.4j				
	Mean	10.7	10.2					
Iso-butyrate	0	1.3	1.3	1.29j	0.08	0.889	0.001	0.899
	4	0.9	0.9	0.89k				
	8	0.8	0.8	0.81k				
	Mean	1	1					
Valerate	0	2.7	2.3	2.46	0.20	0.363	0.199	0.409
	4	2.7	2.6	2.65				
	8	2.8	2.9	2.81				
	Mean	2.7	2.6					
Iso-valerate	0	1.5	1.9	1.71j	0.19	0.15	0.001	0.755
	4	0.9	1.2	1.01k				
	8	0.9	1.0	0.97k				
	Mean	1.1	1.3					

Table 4. Effects of Bioflavex on relative quantification ($2^{(-DCt)} \times 10^2$) of *Streptococcus bovis, Selenomomas ruminantium* and *Megasphaera elsdenii*, and over the post-feeding period

T, treatment effect; H, sampling time; T · H, interaction of the treatment and time post-feeding. Within a row, lower case letters indicate a significant interaction (at $P = 0.05$)

Parameter	Time (h)	Treatment		s.e.m.	*P*-value		
		CTR	BF		T	H	T · H
		Relative quantification					
S. bovis	0	0.12	0.12	0.013	0.798	0.017	0.964
	4	0.09	0.08				
	8	0.08	0.08				
M. elsdenii	0	0.009	0.002	0.018	0.055	0.023	0.193
	4	0.018	0.059				
	8	0.027	0.084				
S. ruminantium	0	2.7	3.1	0.309	0.001	0.989	0.054
	4	2.6	3.1				
	8	2.0y	3.7x				

Table 5. Average gas production (mL/g DM) at each measuring interval obtained from culture media using rumen liquor from steers fed high-concentrate diets, unsupplemented (CTR) or supplemented with Bioflavex (BF) or its pure flavonoid components

BF, Bioflavex; NG, naringine; NH, neohesperidine; PC, poncirine; HS, hesperidine; IN, isonaringine; NE, neoeriocitrine; CTR, control. NG, NH and PC are major components where HS, IN and NE are tracer components of flavonoids mixture. Values within a row followed by different letters are statistically significantly different (at $P = 0.05$) among the treatments

Time (h)	Treatment								s.e.m.	*P*-value
	CTR	BF	NG	NH	PC	HS	IN	NE		
2	47.7ab	39.9bc	25.4d	37.3bc	40.2bc	51.8a	43.3abc	35.9c	2.09	0.001
4	92.5ab	85.8bc	83.2bcd	75.7d	81.5cd	96.9a	85.8bc	89.7abc	1.91	0.001
6	126.8abc	119.8cd	120.0bcd	104.8e	113.3de	130.4a	120.2bcd	128.9ab	1.87	0.001
8	153.2ab	143.5cd	149.6bcd	131.0e	142.0d	151.4abc	147.3bcd	159.5a	1.9	0.001
12	173.8ab	162.7c	166.9bc	149.3d	161.2c	168.8abc	165.1bc	179.1a	2.06	0.001

Table 6. ***In vitro*** **fermentation parameters in response to flavonoid mixture (Bioflavex) and its pure flavonoid components**
BF, Bioflavex; NG, naringine; NH, neohesperidine; PC, poncirine; HS, hesperidine; IN, isonaringine; NE, neoeriocitrine; CTR, control; VFA, volatile fatty acid. NG, NH and PC are major components where HS, IN and NE are tracer components of flavonoids mixture. Values within a row followed by different letters are statistically significantly different (at $P = 0.05$) among the treatments

Parameter	Treatment								s.e.m.	*P*-value
	CTR	BF	NG	NH	PC	HS	IN	NE		
Ammonia-N (mg/L)	158.8a	143.8ab	149.0ab	146.1ab	140.5ab	152.5ab	135.9b	150.1ab	10.21	0.045
Lactate (mM)	0.81	0.45	0.55	0.5	0.98	0.48	1.02	1.35	0.37	0.102
VFA (mM)	37.5	34.7	31.9	33.4	33.4	33.4	33	34.6	2.97	0.428
				VFA (mol/100 mol)						
Acetate	58.9a	53.6b	51.3b	52.0b	52.5b	53.2b	51.5b	52.2b	1.68	0.001
Propionate	25.5b	30.3a	33.0a	31.9a	32.4a	30.7a	33.7a	31.8a	1.94	0.001
Butyrate	10.2	10.3	9.8	10.4	9.7	10.6	9.4	10.1	0.69	0.905
Iso-butyrate	1.9	2	2	2	1.8	1.9	1.7	2	0.21	0.302
Valerate	2	2.1	2.1	2.1	2.1	2.1	2.2	2.3	0.13	0.334
Iso-valerate	1.5	1.7	1.7	1.7	1.6	1.7	1.6	1.7	0.35	0.898

Table 7. Effects of Bioflavex on relative quantification of ***Streptococcus bovis, Selenomomas ruminantium*** **and** ***Megasphaera elsdenii*** **over the incubation time**
BF, Bioflavex; NG, naringine; NH, neohesperidine; PC, poncirine; HS, hesperidine; IN, isonaringine; NE, neoeriocitrine; CTR, control. NG, NH and PC are major components where HS, IN and NE are tracer components of flavonoids mixture. Within a row, values followed by different letters are significantly different (at $P = 0.05$)

Parameter	Treatment								s.e.m.	*P*-value
	CTR	BF	NG	NH	PC	HS	IN	NE		
S. bovis[A]	0.015b	0.014b	0.013bc	0.002d	0.005cd	0.014b	0.005cd	0.032a	0.0015	0.001
M. elsdenii[B]	0.8b	4.9a	4.4ab	5.7a	3.1ab	1.1b	3.6ab	1.3b	0.67	0.004
S. ruminantium[A]	0.1b	0.5ab	0.4ab	0.2ab	0.4ab	0.6a	0.5ab	0.1b	0.08	0.010

[A] $2^{(-DCt)} \cdot 10^2$.
[B] $2^{(-DCt)} \cdot 10^6$.