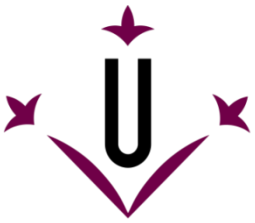

The effect of Bioflavex® and its pure flavonoid components on *in vitro* fermentation parameters and methane production in rumen fluid from steers given high concentrate diets

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Abstract

An *in vitro* assay was designed to analyze the effect of either Bioflavex® (BF) or each of its pure flavonoid components [Neoeriocitrine (NE), Naringine (NG), Isonaringine (IN), Hesperidine (HS), Neohesperidine (NH), Poncirine (PC)] added at 200 µ,g/g dry matter (DM) incubated substrate on rumen fermentation, methane production (CH_4) and microbial population. A treatment without flavonoids was also included as a control (CTR). Rumen liquor harvested from four steers fed with high concentrate diets was used as inoculum in four 72 h incubation series. Two samples were taken at the onset of each incubation series (Time 0), and two bottles per treatment were also opened after 12 h and sampled for pH, NH_3-N, volatile fatty acids (VFA) and microbiology analyses [total bacteria, *Streptococcus bovis*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, total archaea (TA), hydrogenotrophic methanogenic archaea (HMA) and *Methanosarcina spp.* (as acetoclastic

methanogen)] using quantitative PCR. The addition of BF or its flavonoid components mitigated the cumulative gas production ($P<0.01$), except for NE and PC, but no differences ($P>0.10$) were recorded in the gas production rate (mL/h). At 12 h post incubation methane production (mL/g DM) was reduced ($P<0.01$) by flavonoid addition, except for NE and NG, that did not differ from CTR. No changes were detected in total VFA concentration, but flavonoids increased pro- pionate to the detriment of acetate proportion ($P<0.01$). The abundance of HMA population was reduced ($P<0.01$) by BF and its main components (NG and NH). Relative quantification of the lactate producing bacteria *S. bovis* was not affected by the addition of flavonoids except for a significant increase recorded with NE ($P<0.01$), whereas the concentration of the lactate consuming *M. elsdenii* was increased by BF, NG, NH and PC ($P<0.01$). Relative quantification of HMA was clearly inhibited ($P<0.01$) by the addition of flavonoids, this effect being more pronounced with BF, NH and NG. Concentration of *Methanosarcina spp.* was also inhibited by PC, NH, NG and BF ($P<0.01$). Addition of flavonoid substances enhances fermentation efficiency by improving propionate in detriment of acetate production and clearly depressed HMA communities.

Keywords: Flavonoids, *In vitro* incubation, Gas production, Methanogenesis

Introduction

Among other alternatives, dietary addition of flavonoids has been proposed to antibiotic therapies to prevent rumen acidosis and bloat in beef cattle given high concentrate diets (Rhodes, 1996; Broudiscou and Lassalas, 2000; Cushnie and Lamb, 2011). Flavonoids are benzo-l-pyrone derivatives from fruits, vegetables and seeds that have anti-inflammatory, antioxidant and antimicrobial properties (Harborne and Williams, 2000). The effect of plant extracts containing flavonoids on rumen fermentation has been studied *in vivo* (De Freitas et al., 2007; Balcells et al., 2012) and *in vitro* (Broudiscou and Lassalas, 2000; Yaghoubi et al., 2007), promoting changes in pH, propionate proportion and protein degradation. However, results are not conclusive, lacking of a homogeneous response (Broudiscou et al., 2000; Broudiscou and Lassalas, 2000).

Balcells et al. (2012) showed that Bioflavex®, a citrus extract rich in flavonoid substances from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradisi*), was able to exert a favorable activity on rumen environment when growing steers were given a high concentrate ration or under experimental acidosis induction. The flavonoid blend was effective in mitigating ruminal pH decreases and enhancing the molar proportion of propionate and reducing that of acetate.

Changes in volatile fatty acids (VFA) profile hypothetically imply a dihydrogen re-canalization and thus changes in CH_4 synthesis (Demeyer and Van Nevel, 1975), assuming that CH_4 is the major sink of the hydrogen released during VFA synthesis (Ørskov et al., 1968; Yáñez-Ruiz et al., 2010). Previous studies evidenced that the addition of plants extracts rich in secondary compounds such as saponins, tannins, essential oils and also flavonoids reduces rumen CH_4 production (Patra and Saxena, 2010). However, plant extracts are constituted by complex mixtures which action on rumen fermentation could be synergistic or antagonistic depends on the mixture composition (*i.e.*, plant flavonoids mixtures, Broudiscou et al., 2000).

Therefore, the present assay was conducted to analyze the impact of Bioflavex® on rumen fermentation, methane production and microbial population, identifying the specific activity of its pure flavonoid components under *in vitro* conditions using rumen liquid from growing steers fed with high concentrate diets.

Materials and methods

Incubation procedure

The effect of Bioflavex® (BF) and its pure flavonoid components (Hesperidine, HS; Isonaringine, IN; Naringine, NG; Neo- eriocitrine, NE; Neohesperidine, NH and Poncirine, PC) on rumen fermentation were tested against the control (CTR) at 200 µ,g/g dry matter (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, a different one used as inoculum donor for each incubation series. Animals were fed a concentrate ration consisting of 0.90 of a commercial concentrate and 0.10 barley straw (Table 1) offered *ad libitum.* The rumen contents were sampled at (08:00 h) and filtered through a double layer of gauze and used immediately as inoculum at 0.10 of total incubation volume. Four serum glass bottles (120 mL) for each experimental treatment were filled with 80 mL of an incubation solution including rumen inocu- lum and mineral and buffer solutions plus a HCl–cysteine reducing solution, prepared under a CO_2 stream (Mould et al., 2005). In order to approach the fermentation characteristics to practical feeding conditions, a mixture of 600 mg of the same concentrate given to steers, plus 60 mg barley straw was used as substrate. Bottles were sealed with butyl rubber stoppers and aluminum crimps and incubated at 39 ± 1 °C in a shaking water bath for 72 h.

Pressure measurements were determined with a TP704 Manometer (DELTA OHM, Italy) at 2, 4, 6, 8, 12, 24, 48 and 72 h of incubation. Pressure readings were converted to volume by a linear regression established between pressure and known air volumes at an equal incubation temperature. Gas volume at each incubation time was expressed per unit of dry matter (DM). At 12 h post incubation, after gas pressure measurements, a sample (0.1 mL) from the head space gas was taken manually using a gastight syringe (1001SL 1.0 mL SYR $22/2^{11}/2$ L, Hamilton syringe Gastight®, Nevada, USA) and immediately analyzed for methane concentration.

Sampling and analyses

Two samples from the stock solution were taken at the onset of each incubation set (Time 0) for analyses. Besides, two bottles per treatment were opened after 12 h of incubation, their pH determined (pH-meter 2000 Crucible, Crucible Instruments, Barcelona, Spain) and 12 mL of the incubation media were weighed, immediately frozen in liquid nitrogen and stored at −80 °C for subsequent molecular analyses. The remaining content was filtered through a metal sieve (1 mm pore size) and sampled for ammonia nitrogen (2 mL over 0.8 mL of 0.5 N HCl) and VFA (4 mL on 1 mL solution made up with 20 mL/L ortho-phosphoric acid and 2 g/L of 4-methylvaleric acid, in distilled water) concentration. Samples were immediately frozen (−20 °C) until further analyses. The remaining two bottles per treatment were incubated for gas production measurements until the end of the incubation period (72 h).

The concentrate and straw used as substrates were analyzed in duplicate following the procedures of AOAC (2005). The DM content was determined by oven drying at 105°C until a constant sample weight (ref. 934.01), ash content was determined by incineration on muffle furnace at 550 °C for 4 h (ref. 942.05) and crude protein (CP) was analyzed by the Kjeldahl method (ref. 976.05). The proportion of neutral detergent fiber (NDF) was determined according to Van Soest et al. (1991) procedures, using alpha amylase but not sulfites, and discounting ashes from the residue. Ammonia-N concentration was determined by the Chaney and Marbach (1962) method after sample centrifugation (25,000 × *g*, 20 min). The VFA concentration and the molar VFA profile were determined by gas chromatography according to the technique proposed by Jouany (1982), using a capillary column (BP21, 30 m × 0.25 mm ID × 0.25 µ,m, DE, USA). Methane concentration was calculated from the peak to area ratio using a standard gas (CH_4; 99.995% purity, C45, Carburos Metalicos, Spain) as a reference. Different head space volumes of the standard mixture (0.1, 0.3, 0.5, 0.7 and 0.9 mL) were manually injected into the gas chromatograph to obtain a standard curve.

DNA extraction, real time-PCR analyses

The DNA was extracted from samples using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions. Real time PCR (qPCR) was used to quantify the numbers of bacteria and hydrogenotrophic methanogenic archaea (HMA), which were expressed as DNA concentration on Log_{10} of gene copy number/g sample. Specific primers (Øvreås and Torsvik, 1998; Denman et al., 2007) were used to determine the

relative abundance of *Streptococcus bovis*, *Selenomonas ruminantium* and *Megasphaera elsdenii* in relation to the total bacteria and HMA, and *Methanosarcina spp.* (acetoclastic methanogen archaea) in relation to total archaea. The relative quantification was carried out as the $2^{-.6.Ct}$ (Livak and Schmittgen, 2001). Analyses were performed on a CFX96 Touch real-time PCR detection system (BioRad, Laboratories Inc., Hercules, CA, USA). The primer sets and qPCR conditions are described in Table 2.

Calculations and statistical analysis

The rate of gas production was estimated from the cumulative gas production at incubation times varying from 2 to 72 h by means of nonlinear regression. The pattern of gas production was fitted iteratively (SAS NLIN program) to the model proposed for rumen degradability by McDonald (1981), modified as: $y = a(1 - e^{-b(t - c)})$, where y is the cumulative gas production at a given time (mL); a is the potential cumulative gas production (mL); b is the rate of gas production (mL/h); t is the time of fermentation (h); and c is the discrete lag time (h). The four sets of incubation series (batches) were separately conducted using a completely randomized block with eight treatment factors (7 flavonoid sources plus a CTR) occurring in four different blocks (incubations). Each incubation series was considered as the experimental unit and within each incubation series, all traits (including gas, pH, VFA, NH_3 and microbial abundances) were analyzed in duplicate, with two samples taken from the original stock solution and with two bottles sampled after 12 h. Tukey multiple comparison procedure was applied to all treatments at an alpha value of 0.05. Computations for the repeated measurement were performed using the mixed procedure of SAS (Inst. Inc., Cary, NC). The model included block (considered as a random effect), treatment, time of incubation (considered as repeated measures) and their interactions as fixed factors. Significant differences and tendencies were declared at $P<0.05$ and $P<0.10$, respectively.

Results

In vitro gas and methane production values are presented in Table 3. The addition of BF or any of its flavonoid components to the culture media reduced the cumulative gas production ($P<0.01$), except for NE and PC. No differences ($P>0.10$) were observed in the gas production rate (mL/h) in relation to CTR. Methane production (mL/g DM; at 12 h post incubation) was also reduced ($P<0.01$) by flavonoid supplementation, except for NG and NE, that did not differ from CTR. When methane production at 12 h incubation was expressed in relation to VFA concentration (Table 3) no changes were observed compared to CTR except for NH ($P<0.05$). The CH_4 to total gas production ratio (v/v) was also calculated, NE recorded the greater methane proportion to produced gas ($P<0.01$) whereas BF and PC decreased ($P<0.01$) methane concentration in relation to CTR.

The pH values were maintained between 6.7 and 6.8 during the 12 h incubation period and no treatment differences were observed ($P>0.10$). No differences treatments were recorded ($P>0.10$) on the concentration of ammonia and total VFAs (Table 4). However, BF and the flavonoid compounds supplementation, except for HS, altered the VFA profile in comparison to CTR, reducing ($P<0.01$) the molar proportion of acetate and increasing ($P<0.01$) that of propionate. Adding the bottles with flavonoids did not affect butyrate proportion ($P>0.05$).

Absolute concentrations of total bacteria and HMA, together with the relative quantification of the specific rumen bacteria, are presented in Table 5. Addition of flavonoid substances did not change the total bacteria concentration in relation to the CTR. Concentration of HMA community was reduced ($P<0.01$) with BF and its main flavonoid components (NG and NH) where the rest of flavonoid substances did not show any significant effect compared to CTR.

Flavonoids did not affect the relative quantification of the lactate producing bacteria *S. bovis* in the incubation media, although it was enhanced by NE in relation to the CTR (0.096 *vs.* 0.010; $P<0.05$). Relative quantification of the lactate con- suming species *M. elsdenii* was increased ($P<0.01$) by addition of flavonoids, except for IN, HS and NE, whereas no differences were observed in the case of *S. ruminantium* in relation to CTR. A clear inhibition of flavonoids on the relative abundance of HMA was observed ($P<0.01$), being more pronounced with BF, NH and NG. Relative abundance of *Methanosarcina* (as acetoclastic methanogenic archaea) was reduced by the addition of PC, NH, NG and BF (Table 5; $P<0.05$).

Discussion

In a previous work (Balcells et al., 2012) we observed that the addition of Bioflavex® was effective in mitigating ruminal pH reductions in heifers experimentally subjected to induced acidosis. Apparently, BF created favorable conditions for lactate- consuming microorganisms, but its effect on the whole rumen population remained unclear. In the present approach, the addition of flavonoids to the *in vitro* culture media reduced the volume of gas production. Gas production is an index of microbial fermentative activity, although changes in VFA proportion may cause small variations on gas volume (Beuvink and Spoelstra, 1992) therefore our results on gas production would suggest an unspecific activity of the flavonoid extracts (BF and its main component NG) against microbial activity. Scarce evidence of flavonoid activity on rumen microbial fermentation does exist in available literature. Mirzoeva et al. (1997) reported that NG depresses activity of *Escherichia coli*, its effect being mediated through the disruption of proton motive force and inhibition of bacterial motility. Besides, NG is degraded to its aglycone (Naringein) in the rumen of sheep (Gladine et al., 2007) and acts against the fibrolytic *Ruminococcus albus* (Stack et al., 1983) or further degrades to phenylacetic acid which has demonstrated antimicrobial properties (Winter et al., 1989).

The addition of flavonoids modify the environment of the culture media, confirming an small but significant increase in activity of lactating-consuming bacteria (*M. elsdenii*, Table 5) and this effect seems to be promoted by the presence of NG and NH as the main components of BF. However, NE, IN and HS did not exert positive effect on lactate-utilizing bacteria, and NE further promoted the growth of *S. bovis*, identified as a lactate-producing microorganism responsible of cases of acute and sub-acute acidosis (Nagaraja and Titgemeyer, 2007). Therefore, flavonoid compounds seem to exert antimicrobial properties, although their mechanism depends on their chemical nature. Our results agree with the differential activity of flavonoids structures on *in vitro* rumen microbial fermentation reported by Broudiscou et al. (2000).

The addition of BF mixture reduced the volume of CH_4 production (mL/g DM) and its proportion (mL/mL total gas) at 12 h post incubation although the effect of flavonoid addition on total VFA concentration was minor. The presence of flavonoid substances in the media did not reduce total titers of bacteria, nor promoted significant changes in VFA concentration.

In our study, the addition of flavonoids did not change the total VFA concentration in relation to the CTR, but significantly depressed the molar proportion of acetate and increased that of propionate, and it correlated negatively with CH_4 production. This reduction of CH_4 production should be explained by a hypothetical re-canalization of the excess of hydrogen toward a propionate metabolic pathway, as it may occur with other anti-methanogenic compounds (Demeyer and Van Nevel, 1975; McAllister and Newbold, 2008). Therefore, the addition of specific flavonoid substances may alter specifically rumen microbial ecosystem and improve microbial growth efficiency. The specific effect of flavonoids substances on the whole microbial population is scarcely documented, but our results support those previously obtained *in vitro* by Wang et al. (2013), who reported a significant depression of methanogens population by an extract of the plant *Portulaca oleracea*, rich in flavonoids.
Considering that the main proportion of methane synthesized in the rumen environment comes from the activity of HMA population, data shown in Table 5 confirm the specific effect of BF and its main components (mostly NG and NH) on the concentration of HMA. Moreover, there was a tight relation between HMA abundance (Log of *mcrA* gene copies number/g FM) and the extent of CH_4 inhibition, whereas BF and NG and NH reduced HMA in both absolute and relative (in relation to total archaea) terms.

Bioflavex® includes different amounts of the tested pure flavonoids and its effect on the inhibition of both CH_4 emission and HMA abundance was, in general similar to those observed for its pure corresponding components, although their specific concentration in the blend were much lower. This study suggests that either flavonoids may have a threshold level for their activity, that would be below the BF dosage, or that the different flavonoid substances in the blend may act synergetic in relation to CH_4 emission.

Our results agree well with previous reports showing the *in vitro* inhibitory activity of pure flavonoid, such as Naringine and Quercitine (Oskoueian et al., 2013) or other polyphenol compounds like 9,10-anthraquinone (Garcia-Lopez et al., 1996) or that from plant extracts rich in flavonoids (Broudiscou et al., 2000; Bodas et al., 2012) on methane production.

It has been suggested that the inhibition of CH_4 occurs through two main mechanisms. Firstly, those compounds that indirectly affect methane formation by interfering or reducing carbon or electron flow in the microbial food chain. In this approach, hydrogen would not accumulate and propionate would increase at the expense of acetate and butyrate, as it has been previously mentioned in this discusion. An example of this should be the ionophore-like compounds, that act against bacteria that produce hydrogen and carbon dioxide as

precursors for methanogenesis (Chen and Wolin, 1979). Alternatively, some methane inhibitors may be toxic to methanogens (*i.e.*, oxygen, carbon dioxide, fatty acids). Authors are not aware of reports describing the anti-methanogenic mechanism of NG or NH as the main flavonoid components of Bioflavex® mixture. From our data, it cannot be determined if the methane inhibition by flavonoid substances occurs through a clear depres- sion of abundance of methanogenic archaea, or, on the contrary, it is carried out through a ionophore-like mechanism.

However, the fact that the presence of flavonoids in the incubation media also depresses the acetoclastic methanogenic archaea *Methanosarcina* seems to suggest a hypothetical toxicity of either flavonoids or its degradation metabolites against the methanogenic archaea populations.

Conclusions

The present assay showed the activity of the commercial citrus extract of flavonoids blend (Bioflavex®) and its primary components to partially inhibit methane production in an *in vitro* fermentation system. Addition of flavonoid substances reduced gas production as an index of microbial fermentative activity in high concentrate diets. Flavonoids cause changes in the fermentation end products and also alter the concentration and composition of lactate-utilizing bacteria (*M. elsdenii*) and methanogenic archaea, although differences among the specific flavonoids have also been detected. Further research is needed to establish a long-term efficacy and to elucidate the potential interaction among flavonoid substances, to facilitate the use of plant extracts rich in different flavonoid compounds.

Conflicts of interest

There are no conflicts of interest.

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Table 1
Ingredient and chemical composition of the experimental diet.

	Concentrate	Barley straw
Ingredients (g/kg)		
Corn grain	350	
Barley grain	250	
Soybean meal (44%)	100	
Wheat bran	60	
Sunflower meal (30%)	80	
Gluten feed (20%)	70	
Sugarbeet pulp	25	
Palm oil	35	
Calcium carbonate	13	
Bi-calcium phosphate	8	
Sodium chloride	3	
Mineral and vitamin premix	4	
Sepiolite	2	
Chemical composition (g/kg DM)		
Organic matter	932	934
Crude protein	154	38
Neutral detergent fiber	293	744

Mineral and vitamin premix, [IU/kg]: Vitamin A, 5.000; 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 qPCR used in the Experiment.

Target	Authors		Primer
† Total bacteria	Maeda et al. (2003)	F	5^1 -GTGSTGCAYGGYTGTCGTCA-3^1
		R	5^1 -ACGTCRTCCMCACCTTCCCC-3^1
† *Selenomonas ruminantium*	Tajima et al. (2001)	F	5^1 -TGCTAATACCGAATGTTG-3^1
		R	5^1 -TCCTGCACTCAAGAAAGA-3^1
† *Streptococcus bovis*	Tajima et al. (2001)	F	5^1-CTAATACCGCATAACAGCAT-3^1
		R	5^1 -AGAAACTTCCTATCTCTAGG-3^1
† *Megasphaera elsdenii*	Ouwerkerk et al. (2002)	F	5^1 -GACCGAAACTGCGATGCTAGA-3^1
		R	5^1 -CGCCTCAGCGTCAGTTGTC-3^1
¥ Hydrogenotrophic Methanogens	Denman et al. (2007)		5^1-TTCGGTGGATCDCARAGRGC -3^1
		R	5^1-GBARGTCGWAWCCGTAGAATCC-3^1
¥ *Methanosarcina spp.*	Franke-Whittle et al. (2009)		5^1 -CCTATCAGGTAGTAGTGGGTGTAAT-3^1
		R	5^1-CCCGGAGGACTGACCAAA-3^1
¥ Total *Archaea*	Øvreås and Torsvik (1998)		5^1 -AGGAATTGGCGGGGGAGCA-3^1
		R	5^1 -BGGGTCTCGCTCGTTRCC-3^1

† 1 × (95 °C 10:00 min), 40 × (95 °C 00:15 min, 60 °C 00:10 min, 72 °C 00:55 min).
¥ 1 × (95 °C 10:00 min), 40 × (95 °C 00:15 min, 57 °C 00:10 min, 72 °C 00:55 min).

Table 3
Estimated values for accumulative gas production (*a;* mL/g DM) and rate of gas production (*b*; mL/h) and lag time for gas production (h) obtained in cultures media using rumen liquor from four steers fed high concentrate diets, supplemented with Bioflavex® or its flavonoids components or without (CTR) along with methane production values (mL/g DM) and ratios at 12 h post incubation.

Items	Treatments								SEM	P value
	BF	HS	IN	NG	NE	NH	PC	CTR		
Gas production										
Total gas (*a*)	253^{b}	250^{b}	245^{b}	252^{b}	262 ^{ab}	251^{b}	272^{ab}	283^{a}	6.33	<0.01
Fractional rate (*b*)	0.076	0.083	0.091	0.097	0.092	0.08	0.078	0.091	0.005	0.172
Lag time (*c*)	−0.101	−0.045	−0.494	−0.027	−0.123	−0.054	−0.863	−0.12	0.26	0.26
Methane production										
12 h	16.02^{c}	17.49^{bc}	17.73^{bc}	20.60^{ab}	24.01^{a}	16.91^{bc}	17.54^{bc}	21.82^{a}	0.742	<0.01
Methane ratio (mL/mL total gas)										
12 h	0.106^{d}	0.111^{cd}	0.108^{cd}	0.123^{b}	0.139^{a}	0.109^{cd}	0.106^{d}	0.116^{bc}	0.0015	<0.01
mL/mmol VFA (12 h incubation)										
	0.490^{ab}	0.535^{ab}	0.539^{ab}	0.590^{ab}	0.672^{a}	0.477^{b}	0.532^{ab}	0.590^{ab}	0.045	0.053

Different superscripts (a, b, c, d) denote statistical differences (P<0.05) among treatments. Bioflavex® (BF), Hesperidine (HS), Isonaringine (IN), Naringine (NG), Neoeriocitrine (NE), Neohesperidine (NH), Poncirine (PC) and Control (CTR).

Table 4
In vitro pH and concentration of ammonia–N (NH_3–N) and volatile fatty acids (VFA) with the flavonoid mixture and its components.

Items	Treatments								SEM	P value
	BF	HS	IN	NG	NE	NH	PC	CTR		
pH	6.79	6.78	6.75	6.76	6.77	6.76	6.76	6.77	0.015	0.534
NH_3-N (mg/L)	154	156	140	151	160	147	139	153	11.7	0.110
VFA, m*M*	32.9	33.1	33.5	35.3	36.3	35.6	34.5	37.5	2.91	0.396
VFA, mol/100 mol										
Acetate	52.8^{b}	53.1^{b}	52.2^{b}	52.2^{b}	52.8^{b}	52.6^{b}	53.6^{b}	58.7^{a}	1.79	<0.01
Propionate	30.7^{a}	30.5^{ab}	33.1^{a}	32.7^{a}	31.0^{a}	31.6^{a}	32.0^{a}	26.3^{b}	2.09	<0.01
Butyrate	10.7	10.6	9.16	9.27	10.4	10.3	9.29	9.30	0.71	0.520
Iso-butyrate	1.93	1.93	1.93	1.93	1.87	1.94	1.58	1.96	0.248	0.319
Valerate	2.20	2.10	2.05	2.30	2.32	2.10	2.05	2.00	0.170	0.239
Iso-valerate	1.69	1.69	1.55	1.61	1.70	1.59	1.50	1.67	0.331	0.294

Different superscripts (a, b) denote statistical differences (P<0.05) among treatments.
Bioflavex® (BF), Hesperidine (HS), Isonaringine (IN), Naringine (NG), Neoeriocitrine (NE), Neohesperidine (NH), Poncirine (PC) and Control (CTR).

Table 5
Effects of the flavonoid mixture and its components on absolute quantification (Log gene copies/g FM) of total bacteria and hydrogenotrophic methanogens, and relative quantification of *Streptococcus bovis*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, hydrogenotrophic methanogens and *Methanosarcina Spp.*

Items	Treatments								SEM	P value
	BF	HS	IN	NG	NE	NH	PC	CTR		
Absolute quantification										
Total bacteria	10.17	10.49	10.41	10.50	10.28	10.70	10.52	10.97	0.153	0.117
Hydrogenotrophic methanogens	7.60^{bc}	8.10^{abc}	8.07^{abc}	7.42^{c}	8.44^{ab}	7.56^{bc}	7.85^{abc}	8.73^{a}	0.169	<0.01
Relative quantification										
S. bovis†	0.024^{b}	0.009^{bc}	0.005^{bc}	0.014^{bc}	0.096^{a}	0.002^{c}	0.003^{bc}	0.010^{bc}	0.004	<0.01
S. ruminantium†	0.731	0.573	0.189	0.219	0.104	0.222	0.301	0.147	0.124	0.062
M. elsdenii‡	4.96^{b}	1.12^{d}	1.73^{d}	5.83^{ab}	1.69^{d}	6.26^{a}	3.82^{c}	0.760^{d}	0.178	<0.01
Hydrogenotrophic methanogens†	2.72^{c}	9.74^{bc}	7.32^{bc}	3.37^{c}	14.21^{b}	2.99^{c}	7.33^{bc}	25.5^{a}	1.90	<0.01
Methanosarcina Spp.†	1.52^{c}	1.81^{bc}	2.15^{abc}	1.36^{c}	4.12^{a}	1.05^{c}	0.780^{c}	3.75^{ab}	0.381	0.002

Different superscripts (a, b, c, d) denote statistical differences (P<0.05) among treatments.
Bioflavex® (BF), Hesperidine (HS), Isonaringine (IN), Naringine (NG), Neoeriocitrine (NE), Neohesperidine (NH), Poncirine (PC) and Control (CTR).

† $2^{(-.6.Ct)} \times 10^{2}$.

‡ $2^{(-.6.Ct)} \times 10^{6}$.