Universitat de Lleida

Study the effect of inclusion of feed flavonoid substances on animal performance and ruminal fermentation in calves

Ahmad Reza Seradj

Dipòsit Legal: L.236-2015
http://hdl.handle.net/10803/288291

Universitat de Lleida

Agricultural and Food Science and Technology

(Ciència i Tecnologia Agrària i Alimentària)

Department of Animal Production

Study the effect of inclusion of feed flavonoid substances on animal performance and ruminal fermentation in calves

(Estudio del efecto de la inclusión en el pienso de sustancias flavonoides sobre los índices productivos y fermentación ruminal en terneros)

By:

Ahmad Reza Seradj

Directed By:

Joaquim Balcells Terés

A thesis presented for the degree of Doctor of Philosophy at the University of Lleida

January 2015

ACKNOWLEDGEMENTS

Credit is due to my supervisor, Prof. Joaquim Balcells who has made invaluable contributions to the work through his interest, advice and constructive criticism. His effort is sincerely appreciated.

I am indebted to Prof. Manuel Fondevila, who generously gave his assistance and helpful discussion to my work. I am grateful to Dr. Daniel villalba for his advice on the statistical analysis.

I would like to thank Dra. Montserrat Llovera and Dra. Teresa Capell for their help and kindness.

I am grateful to Prof. Diego P. Morgavi, Prof. Cecile Martin and Dr. Matheiu Silberberg from Institut national de la recherche agronomique (INRA-UMRH-DIMA) for receiving me during my sabbatical leave in Clermont Ferrand, France, which was an honor for me to work with them even for a short period of time.

Thanks are due to Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) and the Government of Catalonia for their grants for universities and research centres for the recruitment of new research personnel (FI-DGR 2011), their support from new investigators is also gratefully acknowledged.

Thanks are due to my friend Dr. Henris Morazan who helped me a lot during my PhD period.

Many more members of the department of Animal Production have helped me in one way or another. Their names are not in this list, but in my mind.

DEDICATION

I dedicate my PhD thesis to my family. A special feeling of gratitude to my parents whose words of encouragement and push for tenacity ring in my ears and they were always there for me. My sister Afsaneh has never left my side and is very special.

Ahmad Reza Seradj

Lleida, January 2015

Science may set limits to knowledge, but should not set limits to imagination

Bertrand Russell
(May 18th, 1872 – February 2nd, 1970)

SUMMARY

The first experiment was designed to study the effects of an extract of plant flavonoids (Bioflavex®) on rumen fermentation once experimental acidosis was induced. After acidosis induction rumen VFA and lactate concentration increased whereas acetic acid decreased ($P < 0.01$). Bioflavex® addition buffered rumen pH and improved the abundances of lactate-consumer *M. elsdenii* ($P < 0.05$). In the second experiment, 48 Fleckvieh heifers were used to evaluate the effect of Bioflavex® on the performance and rumen fermentation. Bioflavex® addition did not affect average daily gain, feed consumption or feed conversion ratio. Rumen pH and molar proportions of propionate were greater and acetate proportion was lesser in the treated heifers. The third and fourth experiments, (*in vivo* and *in vitro*) were designed to confirm in a more controlled assay the impact and extension of Bioflavex® addition on rumen fermentation characteristics. In the *in vivo* experiment, 8 Friesian calves fitted with a rumen canula fed basal concentrate (CTR) or CTR supplemented with Bioflavex®. No differences were observed in concentrate and straw intake, average daily gain or feed conversion ratio between treated and CTR calves. Bioflavex® addition improved pH values and molar proportion of propionate, whereas reduced lactate concentration and improved the relative abundance of lactate-consuming microorganisms. In the fourth *in vitro* experiment, Bioflavex® and its main components (i.e: Naringine, Neohesperidine and Poncirine) were added to the incubation medium, with the un-supplemented substrate as control. Bioflavex® and its main ingredients as additives reduced the volume of gas produced and the molar proportion of acetate and increased that of propionate. Poncirine reduced the relative abundance of *S. bovis*, whereas Neohesperidine and Bioflavex® increased the relative abundance of *M. elsdenii*. In the fifth trial, an *in vitro* assay was designed to analyze the effect of Bioflavex® and its pure flavonoid components on methanogen population and methane production. Additives mitigated the cumulative gas production, except for Neoeriocitrine and Poncirine, but no differences were recorded in the gas production rate. Methane production was reduced by flavonoids addition, except for Neoeriocitrine and Naringine. Flavonoids increased the molar proportion of propionate to the detriment of acetate. The abundance of hydrogenotrophic methanogenic archaea was reduced by Bioflavex® and its main components. Relative abundance of the lactate producing bacteria was not affected by the addition of flavonoids except for Neoeriocitrine, whereas the concentration of the lactate consuming was increased by the addition of Bioflavex® and its main components.

RESUM

El primer experiment es va dissenyar per estudiar l'efecte d'un extracte cítric ric en flavonoides (Bioflavex®) a la fermentació al rumen, una vegada s'havia induït experimentalment una situació d'acidosis metabòlica. Després de la inducció la concentració d'AGV i lactat va incrementar i la d'acètic es va reduir ($P<0.01$). La adició de Bioflavex® tamponà el nivell de pH i va incremental el títols de *M. elsdenii* com a bacteria representativa de les consumidores de lactat. En el segon experiment, es va utilitzar 48 vedelles Fleckviek per a avaluar l'efecte de Bioflavex en el rendiment productiu i en la fermentació del rumen. L'adició de Bioflavex® no va afectar al guany mitjà diari, al consum d'aliment ni a l'índex de conversió. El pH del rumen i les proporcions molars de propionat van ser superiors i la proporció d'acetat va ser menor en les vedelles tractades. En el tercer i quart assaig, es van realitzar experiments *in vivo* i *in vitro* per a confirmar els efectes de Bioflavex® en les característiques de la fermentació ruminal. En l'experiment *in vivo*, a 8 vedells Friesian amb canulats al rumen se'ls va subministrar un pinso control (CTR) o CTR suplementat amb Bioflavex®. No es van observar diferències en la ingesta de pinso ni palla, en el guany mitjà diari ni en l'índex de conversió entre vedells tractats i no. Bioflavex® va millorar els valors de pH i la proporció molar de propionat, mentre que va reduir la concentració de lactat i va millorar l'abundància relativa de microorganismes consumidors de lactat. En l'experiment *in vivo*, Bioflavex® i els seus principals components com naringina, neohesperidina i poncirina es van afegir al medi d'incubació, amb el substrat sense suplements com a control. Els additius van reduir el volum de gas produït, així com la proporció molar d'acetat, i van incrementar la de propionat. Poncirine va reduir la quantificació relativa de *S. bovis*, mentre que Neohesperidine i Bioflavex van incrementar la quantificació relativa de *M. elsdenii*. En el cinquè assaig, es va dissenyar una prova *in vitro* per a analitzar l'efecte de Bioflavex® o de cada un dels seus ingredients sobre la fermentació del rumen, en la producció de metà i la població microbiana. Els additius van mitigar la producció de gas acumulativa, a excepció de Neoriocitrine i Poncirine, però no es van registrar diferències en l'índex de producció de gas. La producció de metà es va reduir amb l'addició de flavonoides, a excepció de Neoriocitrine i Naringine. Els flavonoides van incrementar el propionat en detriment de la proporció d'acetat. L'abundància d'hidrogenotròfiques metanogenes archaea es va reduir amb l'adició de Bioflavex® i els seus principals components. La quantificació relativa de les bactèries productores de lactat no es va veure afectada per l'addició de flavonoides, a excepció de Neoeriocitrine, mentre que la concentració de les consumidores de lactat va ser incrementada per l'adició de Bioflavex® i els seus principals components.

RESUMEN

En el primero experimento fue diseñado para estudiar el efecto de un extracto cítrico rico en flavonoides (Bioflavex®) sobre la fermentación ruminal cuando se induce experimentalmente un proceso de acidosis. Tras la inducción las concentraciones de AGV y lactato incrementaron mientras que la proporción de acético se redujo ($P<0.01$). La adición de Bioflavex® tamponó los niveles de pH y mejoró los títulos de las bacterias consumidoras de lactato. En el segundo, se utilizaron 48 novillas Fleckviek para evaluar el efecto de Bioflavex® en su rendimiento productivo y fermentación ruminal. La adición de Bioflavex® no afectó a la ganancia media diaria, consumo de alimento ni al índice de conversión. El pH del rumen y las proporciones molares de propionato fueron superiores y la proporción de acetato fue menor en las novillas tratadas. En el tercer y cuarto experimentos, *in vivo* e *in vitro*, *se* diseñaron para confirmar los efectos de Bioflavex® sobre las características de la fermentación. En el experimento *in vivo*, a 8 terneros Friesian canulados en el rumen se les suministró un pienso control (CTR) o CTR con Bioflavex®. No se observó diferencias en la ingesta de pienso ni paja, ganancia media diaria ni en el índice de conversión entre terneros tratados y CTR. La adición de Bioflavex® mejoró los valores de pH y la proporción molar de propionato, mientras que redujo la concentración de lactato y mejoró la abundancia relativa de microorganismos consumidores de lactato. En el experimento *in vitro*, Bioflavex® y sus principales componentes como naringina, neohesperidina y poncirina se añadieron al medio de incubación, con el sustrato sin suplementos como control. Los aditivos redujeron el volumen de gas producido así como la proporción molar de acetato, e incrementaron la de propionato. Poncirina redujo la cuantificación relativa de *S. bovis*, mientras que Neohesperidina y Bioflavex® incrementaron la proporción relativa de *M. elsdenii.* En el quinto ensayo, se diseñó una prueba *in vitro* para analizar el efecto de Bioflavex® o de cada uno de sus componentes flavonoides puros en la fermentación ruminal, en la producción de metano y la población microbiana. Los aditivos mitigaron la producción de gas acumulativa, a excepción de Neoriocitrina y Poncirina, pero no se registraron diferencias en el índice de producción de gas. La producción de metano se redujo con la adición de flavonoides, a excepción de Neoriocitrina y Naringina. Los flavonoides incrementaron el propionato en detrimento de la proporción de acetato. La abundancia de hidrogenotróficas metanogenas archaea se redujo con la adición y sus ingredientes. La cuantificación relativa de las bacterias productoras de lactato no se vio afectada por la adición de flavonoides, a excepción de Neoeriocitrina, mientras que la concentración de las consumidoras de lactato fue incrementada por Bioflavex® y sus principales ingredientes.

CHAPTER 1

GENERAL INTRODUCTION

High-concentrate diets can cause rumen fermentation dysfunctions such as rumen acidosis or bloat (Beauchemin and Buchanan-Smith, 1990). The inclusion of antibiotics (e.g., monensin) in the diet appears to reduce the incidence of those rumen dysfunctions; however, antibiotics as feed additives were banned by the European Community (European Communities, 2003) then, flavonoids have been proposed as alternatives to antibiotic therapies (Broudiscou and Lassalas, 2000; Rhodes, 1996). 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, anti-oxidant, and anti-microbial properties (Harborne and Williams, 2000). In relation to the anti-microbial properties of flavonoids extracts, the applications of flavonoids extracts to 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 continuous rumen culture system the flavonoids modified fermentation conditions (pH, propionate proportion, protein degradation), although the results were not always homogeneous (Broudiscou and Lassalas, 2000; Broudiscou et al., 1999).

Losses as methane (CH_4) have been considered as a valid index of rumen microbial fermentation inefficiency, changes in VFA profile, i.e improvement in the molar proportion of propionate to the expense of acetate, hypothetically implied, a dihydrogen re-canalization and thus reduction in CH_4 synthesis (Demeyer and Van Nevel, 1975; McAllister and Newbold, 2008) assuming that CH_4 is the major sink of the hydrogen released during VFA synthesis (Orskov et al., 1968; Yáñez-Ruiz et al., 2010). Moreover it is necessary to remark that CH_4 is a potent greenhouse gas which contributes to global warming and is 25 times more toxic than CO_2 (Solomon et al., 2012). Previous studies evidenced that the addition of plants extracts rich in secondary compounds such as saponins, tannins, essential oils and also extracts rich in flavonoids reduced rumen CH_4 production (Patra and Saxena, 2010). However, uncertainties in such processes derived from i) mechanism of methane depression are unidentified and ii) the plant extracts are constituted by complex mixtures whose action on rumen fermentation may derive from the synergistic and/ or antagonistic action of the components (*i.e.*, plant flavonoids mixtures, Broudiscou and Lassalas 2000).

Flavonoids (considered as the polyphenol sunstances) also have important anti-oxidant properties (Harborne and Williams, 2000) and actually plant extract blends, are alternatives of antioxidant additives (Basically Vit E) in the food industry. As an example, oregano and thyme oil have been frequently used successfully for food preservation (Aureli et al., 1992; Chouliara and Kontominas, 2006; Hao et al., 1998). Both contain high concentrations of phenolic compounds such as thymol and carvacrol and lower concentrations of *p*-cymene and *γ*-terpinene (Marino et al., 1999). However, the use of plant extracts rich in flavonoids as dietary antioxidants to preserve both the health of animals and the oxidative stability of their products need further investigations, since studies conducted in rodents and human subjects showed that many polyphenols are poorly absorbed, intensively metabolized or rapidly excreted (Manach et al., 2004). Feeding trials conducted in poultry nevertheless reported the beneficial effects of oregano extract in muscle tissue (Giannenas et al., 2003) but the results cannot be directly extrapolated to ruminants because of the bacterial fermentation processes occurring in the rumen which could greatly affect the bioavailability of flavonoids extracts and subsequently their *in vivo* antioxidant capacity.

Bioflavex® (FL; Exquim S.A., Barcelona, Spain) is a blend of natural flavonoid extracts comprising mostly naringine (200 g/kg), which is extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*) (400 g/kg). From the existing literature it was hypothesized that Bioflavex® could exert some potential anti-microbial also anti-oxidant proprieties.

CHAPTER 2
LITRATURE REVIEW

Rumen Ecosystem

The rumen compartment maintains large anaerobic environment composed by several phases, solid, liquid and gaseous with a dry matter content ranging from 6 to 18% (Hungate, 1966), its kinetics and conditions allow the establishment of several symbiotic microbial populations (Van Nevel and Demeyer, 1988). This complex ecosystem is mostly composed of anaerobic bacteria (~10^{11} cells/mL), anaerobic protozoa (~10^{5} cells/mL), anaerobic fungi (~10^{3} cells/mL) and methanogen archaea (~10^{9} cells/mL) as the main groups of microorganisms which anaerobically break down and ferment the ingested plant materials and their interactions among each other bring a steady state to the rumen (Jouany and Morgavi, 2007). Rumen has an important role in digestion and fermentation processes and once a hemostasis occurs in its environment (i.e. normal and constant temperature 39 °C) those rumen inhabitant micro-organisms start to proliferate and growth , transforming the low quality feedstuffs to volatile fatty acids and high quality protein. As they are very dependent to the host animal, alterations which disturb ruminal fermentation processes will alter type and quantity of nutrients absorbed from the gastrointestinal tract.

Rumen digestion and fermentation

Due to this fact that most of plant material is low in fat and protein and nutrients are confined in the cell wall (Vaughan et al., 2011), it is difficult for animal to digest. Microbial cellulolytic enzymes allow breaking down cell structures and utilizing dietary celluloses and other plant cell wall materials, moreover rumen fermentation prior to their stomach is able to convert dietary material into high quality microbial biomass and end products that can be both used by the host animal.

The ruminal fermentation process is slow, time and space consuming, and includes reduction in size of feed particles, degradation of substrate, utilization of substrate by microorganisms, synthesis of microbial biomass, formation of VFA, ammonia (NH_3), methane, and carbon dioxide as end-products of substrate fermentation.

Different micro-organisms are involved in fermentation process, among them some species have enzymatic activities such as amylase i.e. *Prevotella ruminicola*, *Ruminobacter amylophilus*,

Selenomonas ruminantium, *Succinomonas amylophilus* and *Streptococcus bovis* (Cotta, 1992), also amylase activity has been described among protozoa (Cotta, 1988; McAllister et al., 1990) and also fungi (McAllister et al., 1993; Mountfort and Asher, 1988). Moreover, some species of rumen bacteria are able to utilize fermentation end products of the starch fermenting species i.e lactate utilization by *Megasphaera elsdenii* (Owens et al., 1998).

Rumen Cellulose fermentation

Cellulose is a robust polysaccharide structure placed mostly in plant cell wall and only a few bacterial species e.g. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus* and some strains of *Butyrivibrio fibrisolvens* (Forsberg et al., 2000; Selinger et al., 1996; Teather et al., 1997; Wallace, 1994) have the capability to ferment it. The occurrence of these species is crucial to ruminal fermentation hence their absence impede the degrade process of cellulose and obstruct the access of other bacterial population to their required substrates. Ruminal fungi and protozoa can produce to some extent cellulases and xylanases and contribute in rumen fibrolytic activity of the rumen (Gilbert et al., 1992; Trinci et al., 1994).

Bacteria either cellulolytic or non-cellulolytic can degrade hemicelluloses and pectins. The dominant culturable species involved are: *Prevotella ruminicola*, *Eubacterium ruminantium* and *Bacteroides fibrisolvens*. *Lachnospira multiparus* and *Streptococcus bovis* are pectinolytic (Rasmussen et al., 1988).

Rumen ciliates participate in the fermentation of, both structural and non-structural carbohydrates (Williams and Withers, 1991). Some species which are considered as a larger species (i.e. E. maggii, Epidinium ecaudatum caudatum and Ostracodinium obtusum bilobum) have high cellulolytic activities, being able to be colonized rapidly on fibrous material facilitate their presence in the rumen. The smaller Entodinium spp. is mainly starch digesters and appear to have little or no cellulolytic activity (Mackie et al., 2002).

Rumen protein fermentation

Rumen degradable proteins (RDP) and non-protein nitrogen (NPN) in feedstuffs are digested by microbes to obtain N which further will be used by microorganisms to build its own protein structures that represents the main source of digestible protein for the ruminant animal (Castrillo and Balcells, 2002). A fraction of the dietary is able to pass, un-degraded, to the lower gut along

with the microbial protein. In the context, this fraction is often called escaped protein "by-pass protein" or un-degraded dietary protein (UDP) (Castrillo and Balcells, 2002).

Mechanism of protein degradation in the rumen

After the presence of feed particles in the rumen, a large number of different microbial species attach (Brock et al., 1982) to feed particles, to degrade and ferment nutrients, including protein and degrade it to peptides and eventually amino acids (AA) (Wallace et al., 1997).

Resultant peptides and AA are degraded by extracellular microbial peptidases and further deaminated to ammonia, VFA, CO_2 (Castrillo and Balcells, 2002; Tamminga, 1979). A minor fraction of peptides and AA are transported inside microbial cells, ammonia is the main source of N used by rumen microbes. In fact ammonia-N concentration in the rumen can be considered a valid index of the N availability for an adequate rumen microbial growth (Satter and Slyter, 1974). Dietary plant amides, nitrites and nitrates, and endogenous salivary urea (non-protein nitrogen compounds) ferment to form ammonia (Castrillo and Balcells, 2002; Tamminga, 1979).

Ammonia accumulates due to high proteolysis rate which exceeds the rate of utilization by microorganisms in the rumen. This excess of ammonia is absorbed across the rumen wall, converted to urea in the liver, released as blood urea N (BUN), and can be excreted into urine or recycled back to the rumen via saliva or diffusion through the rumen wall. This inefficient recycling of N is energetically costly to the animal when it exceeds a threshold below which the BUN could support more microbial protein synthesis (MPS) (Firkins and Reynolds, 2005).

Proteolytic microorganisms in the rumen

Proteolytic activity cause the degradation of protein to peptide and AA that are further deaminated to NH_3-N (Brock et al., 1982). As the bacteria enzyme activities are 6 to 10 times higher than that of isolated protozoal cells they are known as the most active proteolytic microorganisms in the rumen (Cotta and Russell, 1997). Among the common rumen microbial species *Prevotella ruminicola* has Dipeptidyl aminopeptidase activity which breaks down peptides from larger peptides followed by di-peptidase (Depardon et al., 1996). On the contrary, in many species, including *P. ruminicola*, and particularly in rumen protozoa, di-peptidase cleaves the dipeptide products.

Deamination of amino acids is carried out by a combination of numerous low activity bacteria (i. e. *P. ruminicola*, *Peptostreptococcus anaerobius*, *Clostridium aminophilum*, and *C. sticklandii*) and protozoa and a much smaller number of high-activity species (Wallace, 1996).

Protozoa are not able to use ammonia then their engulfed insoluble protein is later returned to the rumen fluid in the form of soluble protein to supply soluble protein to sustain microbial growth, so they are playing an important role in regulating bacterial N turnover in the rumen (Bach et al., 2005; Dijkstra, 1994).

Rumen starch fermentation

Content and fermentability of starch in cereal grains

As the grains vary in starch composition [wheat 77% followed by corn and sorghum 72% and barley and oats 57 to 58 %; Huntington (1997)] therefore, initial evaluations of starch content, fermentability and their impact on rumen fermentation are required.

Acidosis due to a large engorgement of grains high in readily fermentable starch leads to accumulation of organic volatile fatty acids (i.e VFA; acetic, propionic, butyric but also lactic acid) and can be harmful to the animal and stimulate a particular dysfunction (Owens et al., 1998). Development of acidosis depends on the quantity and the cleavage rate of starch that varies a lot among the grains. In corn, starch sources are embedded in a protein matrix and thus starch granules has less surface exposed for microbial attach, this protein matrix is lower in wheat grains so engorgement in such case may have a severe consequences than corn grain (Huntington, 1997).

Another significant fraction which should be having in mind is the fiber content of the grains, (i.e. wheat grain is high in starch and low in fiber per unit of its weight in relation to barley, INRA 1989) and also the proportion of soluble starch (i.e greater in barley than in corn, Offner et al. 2003). Therefore wheat has greater fermentation rate over barley (Huntington, 1997) and barley over corn (McAllister and Cheng, 1996) then it can be concluded that using different grain may produce different starch digestibility rates (Feng et al., 1995; Tothi et al., 2003) VFA concentration and then rumen acidity (Feng et al., 1995; Yang et al., 1997).

Starch fermentation

Bacteria as the main inhabitant of the rumen have great influence on starch fermentation (Huntington et al., 1996); around fifteen strains of amylolytic bacteria and eight amylolytic

enzymes were identified (Kotarski et al., 1992). Some of them are floating in the rumen liquid while the others need to be attached and make colony on grain particles before endo - and exo-enzymes production. These bacteria have two sorts of enzymes those which are able to hydrolyze α (1-4) and α (1-6) bonds of amylose and amylopectin. Interaction and cooperation between bacteria in the rumen environment is inevitable and necessary to obtain maximum digestion of starch to monosaccharide.

In a study, Cotta (1992) showed that co-culture of *Streptoccocus bovis*, *Butyrivibrio fibrisolvens*, *Bacteriodes ruminicola*, and *Selenomonas ruminatium* led to cross-feeding among bacterial species and eventually greater bacterial growth rates and complete digestion of starch.

The role of protozoa species on starch fermentation is not negligible, they ferment and store the starch and this storage capacity plays an outstanding role to avoid the excess of VFA concentration and regulation of pH and keep it out of reaching to the acute acidosis levels (Cotta, 1992).

Resultant hexoses and pentoses as end products of starch degradation are metabolized to pyruvate, almost exclusively via the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (France and Siddons, 1993). Pyruvate proceeds to acetate or butyrate with acetyl-CoA as an intermediate. Propionate is formed mainly via succinate (randomizing pathway) but an alternative pathway (direct reductive pathway) involves acrylate (France and Siddons, 1993). Readily fermentable grains has greater ruminal digestion and passage rates that leads to higher propionic acid concentration (Hegarty and Gerdes 1998), while this high digestion rate can be also put the rumen in a risk of acidosis due to lower ruminal pH (Owens et al., 1998).

Rumen structural carbohydrates fermentation

Rumen in ruminants (cattle, goats, and sheep) and large intestine in post-gastric fermenters (horse, donkey and elephant) are the digestive compartments that allow herbivores to use fibrous structural carbohydrates, including cellulose, hemicellulose, lignin, and soluble fiber (fructans, pectans, galactans, and beta-glucans) (Trenkle, 2002). Roughage, depends on plant maturity, contains distinct amount of cellulose and other cell wall components (i.e lignin) that results in a complex and heterogeneous structure (Van Soest, 1994).

Cellulolytic and non-cellulolytic rumen inhabitant microorganisms cooperate to form a degradation complex to have symbiosis with the herbivore animal (Flint and Forsberg, 1995).

In the rumen environment degradation process starts with attachment/colonization process of specialized cellulolytic bacteria species *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fiberobacter succinogenes* to the feedstuffs, ruminal fungi participate in the initial colonization of the substrate while protozoa are also engaged at different degree (Akin, 1986).

An initial adaptation period to the substrate along with an input of maintenance energy is required for ruminal fauna and flora because the degradation of roughage supposed to be inefficient, however it has been proved that roughage maintain a more stable rumen environment (Grant, 1997) and this type of diet are able to maintain higher level rumen microbial synthesis efficiency than concentrate/grain diets (AFRC, 1993).

Having in mind that maintain an adequate neutral detergent fiber (NDF) and acid detergent fiber (ADF) as roughage is crucial to keep ruminal pH and maintain physiological condition (Mertens, 1997).

Volatile fatty acids are considered as the end products of carbohydrate fermentation and they vary due to type of dietary carbohydrate, starch produce higher proportion of propionic acid where fibrolytic bacteria that degrade structural carbohydrates are known as major producer of H_2 lead to increase the proportion of acetic and also butyric acid in the fermentation pathway (Johnson et al., 1996). Acetate to propionate ratio is considered to be an index of energy efficiency of the diets where high acetate/ propionate ratio in roughage diets reflex their low efficiency (Van Kessel and Russell, 1996). VFA are liberated at lower rate than starch fermentation.

Detoxification by rumen microbes

Inhabitant microorganism in the rumen have comprehensive symbiosis with their host in metabolize and deactivating toxic ingredients or in another words detoxifying high proportion of native plants that would be toxic for non-ruminant animals (Jones and Lowry, 1984). Some ruminal detoxification, process are summarized in Table 1.

Compound	Modification/activity	Microorganisms involved
Hydrolysable tannin	Ester hydrolysis	*Selenomonas ruminantium, Streptococcus spp.*
	Ring cleavage	*Streptococcus bovis, Syntrophoccus bovis, Coprococcus spp.*
Ferulic and *P*-coumaric acid	Dehydroxylation	*Unknown*
Flavonoid glycosides	Glycoside hydrolysis	*Selenomonas spp.*
	Heterocyclic ring cleavage	*Butyrivibrio spp., Peptococcus spp.*
Condensed and hydrolysable tannin	Tannin tolerance	*Eubacterium oxidoreducens ButyrivibrioVspp. Streptococcus bovis, Prevotella ruminicola*

(Hobson and Stewart (1997); and Mackie et al. (1997)).

Ruminal fermentation and pH regulation

Different issues have impact over rumen-pH, VFA production and absorption through the rumen-reticulum wall and act as regulatory agents. However lactic acid and ammonia production are able to alter the pH values. Rumen microbial microorganisms are always being affected by pH variation but this incidence is not homogeneous for rumen inhabitants, where in acidity cases (low ruminal pH) microorganisms known as fiber fermenters are more sensitive and their abundance decrease at pH below 6 (Kaufmann et al., 1980) where the starch digesters maintain their activity under acidic environment (5.2 to 6.0; Schwartzkopf-Genswein et al. 2004). Protozoa are not excluded to this rule and decrease under acidic environmental condition, such fact can be used as a conventional defaunation procedure using high concentrate feeding system (Whitelaw et al., 1984).

Microbial consortium runs in equilibrium when rumen pH fluctuation occurs between 5.2 to 6.0, however when acidity persists or the pH decrease below the threshold level of 5.2 (Cooper et al., 1998), rumen environment disturbs and moves from a hemostasis condition to a dysfunction or pathological situation, this dysfunction appears initially at the animal's level then goes further to animal metabolism, such situation is not rare especially when animals are under feedlot and receive a lot of readily fermentable starch.

In case of acidity, rumen proteolytic enzymes which their optimum activity pH is in the range of 5.5 to 7.0 (Kopecny and Wallace, 1982) reduce their activity cause decrease in protein degradation (Cardozo et al., 2002). Also, fiber degradation decreases due to decrease in abundance of cellulolytic bacteria then the access of proteolytic bacteria to proteins is reduced, therefore rumen pH also affects protein degradation (Bach et al., 2005; Mould and Ørskov, 1983).

Nowadays animal production tends to move towards intensifications; animals are feeding with high grain diets which are high in energy and protein content, these anthropogenic changes are known as ruminal manipulations, many manipulators were studied over the years, and they have positive effects and also inconveniences and that is why this field of research is quite wide and to some extent dynamic. Recently, plant secondary metabolites are absorbed the researchers' attention due to their natural occurance and their ability to be served as manipulators basically because of their anti-microbial/ anti-oxidative capacity.

Manipulation

Rumen is not an efficient environment and energy losses during the fermentation process are not negligible (Czerkawski, 1969) so considerable efforts have been done to overcome such inconveniences (Demeyer and Van Nevel 1975). The idea is to control some of the metabolic processes in the rumen to make it more efficient using specific feed additives, in that sense the improvement of volatile fatty acids (VFA) production together with a reduction in both rumen ammonia concentration and methane production are considered as desirable changes in rumen environment (Bodas et al., 2012).

Desirable changes in rumen fermentation can be summarized as follows (Nagaraja et al., 1997):

- To enhance beneficial processes
- To minimize, alter or eliminate inefficient processes

- To minimize, alter or eliminate processes that are harmful to the host

Several feeding experiments were designed to observe the improvements on beef production using several additives. Principally, using ionophores for ruminant diets was developed in the mid-1970s. Ionophores (as monensine) control rumen pH values, improve propionate production and reduce protein degradation together with CH_4 production. Its use increased rapidly in US due to its positive effects in beef cattle production.

However, massive widespread use of antibiotic substances to the environment is associated with the potential cross-over resistance to human therapies, together with consumers' demands on food quality and safety required reduction and recently elimination of dietary antibiotic administration in the EC. Removal of antibiotics conventionally used as feed additives (such as ionophores) increased drastically, fermentation pathologies in ruminants and mortality and morbidity rates. The impact of this policy was so great that farm productivity has been altered and challenged and researchers have been forced to develop alternative types of additives such as probiotics, enzymes, essential oils, organic acids and secondary plant metabolites.

Apart from aforementioned objectives of using feed additives to modify the rumen ecosystem, the debate over the emissions of greenhouse gasses from enteric fermentation by livestock has redirected the researches to develop modifiers that can reduce methane production in ruminal fermentation.

Rumen methanogenesis accounts for ~90 % of methane emission of ruminant origin (Murray et al., 1976). Rumen microorganisms are responsible for digestion in the rumen, during the digestion processes under anaerobic conditions along with principle end products (Microbial protein, VFA and ammonia, utilizing by the animal as energy source) some by products in the form of the gas can be produced (CO_2 and methane) which eructated and cannot be utilized (Martin et al., 2010). Methane has energy content around 55.2 MJ/kg (Brouwer, 1965), and loss of energy as a form of methane is about 8 to 10 % of gross energy intake (Johnson and Johnson, 1995). Therefore, mitigation of this unwanted by product contribute to improve rumen fermentation efficiency. Heritable differences in methanogenesis may cause great variation in methane production in dairy cows (Eckard et al., 2010), therefore animal breeding has got an important place (Cottle et al., 2011) but difficulties in genetic selection (required large number of animals) and prediction of the methane production are inconveniences that bias further investigation.

Other strategies to reduce methane production have been developed, Baker (1999) vaccinated sheep to stimulate the animal's immune system to elicit an immune response and distribute antibodies against methanogens. Concentration of antibodies against methanogens increased significantly after vaccination. The same strategy was tested by Wright et al. (2004) and they depressed CH_4 release in sheep for 8% using this immunization strategy, although in both cases the results were scarcely repeatable with subsequent vaccine preparations. It remains unclear whether it is possible to develop a vaccine with persistent effects on methanogens.

Removing ciliates (defaunation) from rumen environment reduced methane release by 13% (Hegarty 1999) although that persistent effect of defaunation on methane emission has not yet demonstrated (Ranilla et al., 2003). Protozoa is responsible for 10 to 20% of rumen methanogens and produce acetate and butyrate as substrates for methanogenesis (Stumm et al., 1982). The defaunation was therefore considered to reduce methane release. To eliminate protozoa from rumen without altering rumen fermentation is not an easy task, decreasing rumen pH induces defaunation but it improves the risk of acidosis appearance. Other strategies are isolating newborns from other ruminants (Faichney et al., 1999) or using synthetic (e.g. copper sulphate or calcium peroxide) or natural compounds (e.g. vitamin A, non-protein amino acids and steroidal hormones) or biological agents (e.g. fungi, bacteriocins) (Hegarty 1999). However, the collateral effects of defaunation on other parameters related to rumen fermentation need to be further clarified (Eugène et al., 2010).

Feeding the animals with starch or other readily fermentable carbohydrate improves propionate over acetate production and this improvement in fermentation efficiency reduces methane production (Johnson and Johnson, 1995). In this sense Beauchemin and McGinn (2005) demonstrated that the percentage of gross energy intake lost as methane in high grains diets is less than 4% compared with 6.5% or roughage based diets.

Methane production from diets mainly based on forage can be vary due to the type and quality (low or high in NDF or ADF) of roughage (Beauchemin et al., 2009). Maintaining the animal with high quality forages can increase voluntary feed intake, which consequently reduces retention time in the rumen (Eckard et al., 2010) and move the digestion of starch and protein in the small intestine which is more efficient comparing to rumen, resulting in a reduced conversion of feed energy to methane (Blaxter and Clapperton, 1965). Other alternative strategies such as secondary plant extracts (e.g. tannins and saponins) have been tested to improve rumen

fermentation efficiency and methane emission (Beauchemin et al., 2008) and existing literature from both "*in vivo*" and "*in vitro*" studies show a clear relationship between the origin and chemical structures and their corresponding mode of action (Patra and Saxena, 2009).

Feed additives that have consistently demonstrated to be able to alter rumen fermentation can be classified in following groups: antibiotics (such as ionophores derivatives), secondary plant compounds (i.e tannins and saponins), organic acids (i.e. fumarate) and lipids (i.e essential oils).

Ionophores as feed additive

Antibiotics as ionophore types (i.e. Monensin, Lasalocid; Lean et al. 2000) play a central role in prevention of digestive disorders caused by specific feeding dysfunctions including acidosis and bloat (Russell and Strobel, 1989). Ionophores showed a specific effect on specific microbial populations; this active selection was reflected in changes in the rumen environment such as increases in rumen propionate instead of acetate and methane production, controlling rumen pH due to the inhibition of lactate-producing bacteria (i.e. *Lactobacillus*, *Butyrivibrio* and *Lachnospira ssp*; Dennis et al., 1981; Nagaraja et al., 1982), reduction in rumen N-NH_3 concentration as the consequences of the reductions in protein degradation due to an active selection over proteolytic species and a general reduction in feed intake without affecting on growth rate (Chiriase et al., 1991). Legislators in Europe banded ionophores from the end of 2006 and they have been removed from diets as growth promoters.

Plants secondary metabolites as feed additive

The term plant secondary metabolite (PSM) is used to describe a group of chemicals present in plants that are not involved in the primary biochemical processes of plant growth and reproduction. These secondary metabolites protect the plants from insect predation or grazing by the herbivores (Hartmann, 2007).

The positive effect of PSM (e.g. saponins, tannins, essential oils, organosulphur compounds and flavonoids) on rumen microbial fermentation and nutritional stress such as bloat or acidosis have been demonstrated in several studies (Patra and Saxena, 2009) also their desirable effects on the productivity and health of animals (Rochfort et al., 2008).

The suppressive effect of saponins on lactate producing bacteria (*S. bovis*) has been reported by Wang et al. (2000). Anti-microbial features of essential oils (EO) were reviewed by (Greathead,

2003) and Benchaar et al. (2007) reported, that EO increase the pH values through a reduction in VFA production, that means EO suppressed fermentation which is consistent with the antimicrobial activity of phenolic compounds i.e. thymol, carvacrol, and eugenol (due to a hydroxyl group in their phenolic structure; Burt, 2004) in the essential oils (Acamovic and Brooker, 2005; Fraser et al., 2007).

Phenolic compounds disturb the cytoplasmic membrane, disrupting the proton motive force, electron flow active transport, and coagulation of cell contents (Burt, 2004) and have a broad spectrum of activity against a variety of both Gram-positive and Gram-negative bacteria (Lambert et al., 2001).

Flavonoids as polyphenolic compounds have similar pattern of action as monensin (Yaghoubi et al., 2007) showing anti-microbial effects, which may also be considered either positive or negative (Cushnie and Lamb, 2011).

Higher rumen pH values were recorded in high grain feed heifers supplemented with flavonoids in relation to the control group which likely due to the beneficial effect of flavonoid in enhancing lactate-consuming microorganisms i.e. *M. elsdenii* (Balcells et al., 2012).

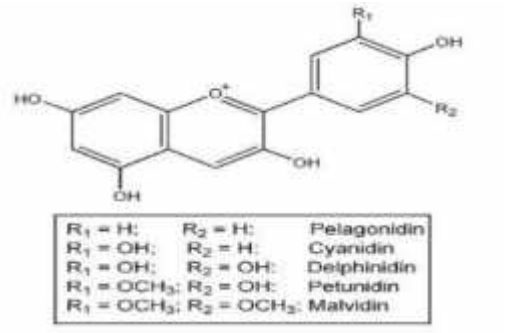
Flavonoids as a potential feed additive

Flavonoids are a group of heterocyclic compounds present in plants and related products, (e.g. propolis; Havsteen 2002) that exert protection against ultraviolet radiation, pathogens, and herbivores (Harborne and Williams, 2000).

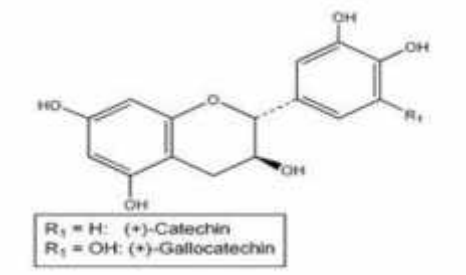
Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Figure 1) and are classified in the following table according to substitutions.

Class	General structure	Examples	Some Common Food Sources
Anthocyanidins	a	Cyanidin, Delphinidin.	Raspberry; cherry (Rice-Evans et al., 1995; Rice-Evans et al., 1996)
Flavanols	b	Monomers (Catechins): Catechin, Epicatechin, Epigallocatechin.	Catechins: Tea (green and white), grapes, apples (Arts et al., 1999; Arts et al., 2000).
	c	Polymers (Proanthocyanidins): Procyanidins, Prodelphinidins.	Proanthocyanidins: apples, berries, red grapes, red wine, condensed tannins (Santos-Buelga and Scalbert, 2000).
Flavanones	d	Hesperetin, Naringenin.	Citrus fruits and juices, e.g., oranges, grapefruits (Rouseff et al., 1987)
Flavonols	e	Quercetin.	Onion, tea (Hertog, 1992; Hertog et al., 1993)
		Kaempferol, myricetin	Grapefruits, black tea cranberry grapes (Spencer et al., 1999)
Flavones	f	Apigenin, Luteolin.	Celery, red peppers, red wine (Hertog, 1992)
Isoflavones	g	Daidzein, Genistein.	Soybean (Reinli and Block, 1996)

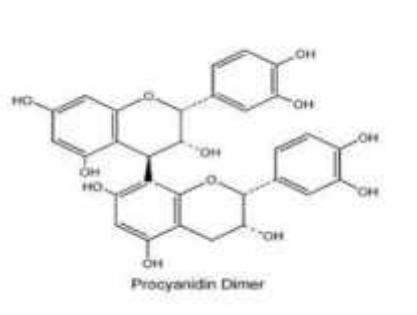
a.


R1 = H; R2 = H; Pelagonidin
R1 = OH; R2 = H; Cyanidin
R1 = OH; R2 = OH; Delphinidin
R1 = OCH3; R2 = OH; Petunidin
R1 = OCH3; R2 = OCH3; Malvidin

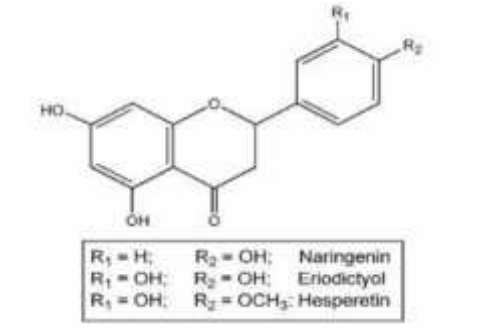
b.


R1 = H: (+)-Catechin
R1 = OH: (+)-Gallocatechin

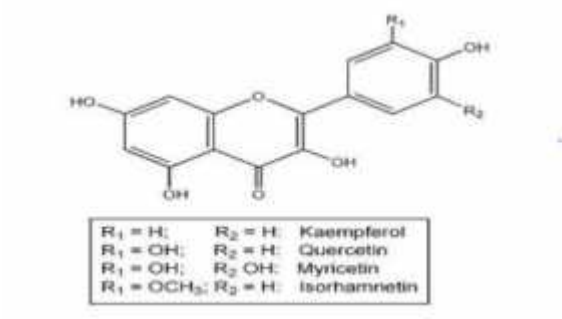
c.


Procyanidin Dimer

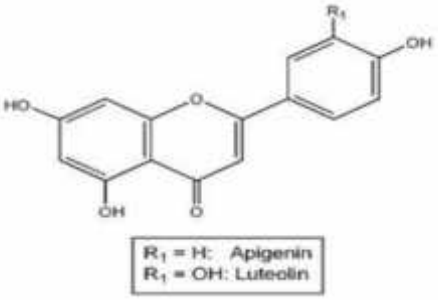
d.


R1 = H; R2 = OH; Naringenin
R1 = OH; R2 = OH; Eriodictyol
R1 = OH; R2 = OCH3; Hesperetin

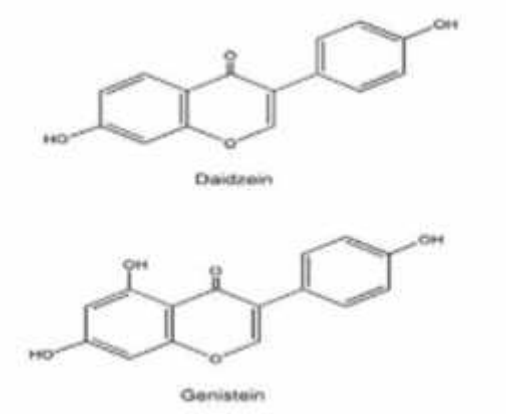
e.


R1 = H; R2 = H; Kaempferol
R1 = OH; R2 = H; Quercetin
R1 = OH; R2 OH; Myricetin
R1 = OCH3; R2 = H; Isorhamnetin

f.


R1 = H: Apigenin
R1 = OH: Luteolin

g.


Daidzein
Genistein

Flavonoids share a common skeleton Diphenyl Pyran (C6-C3-C6), consisting of two phenyl rings (A and B) linked through a pyran C ring (heterocyclic). The carbon atoms in rings C and A are numbered from 2 to 8, and ring B from 2‘ to 6‘ (Figure 1; Heim et al., 2002).

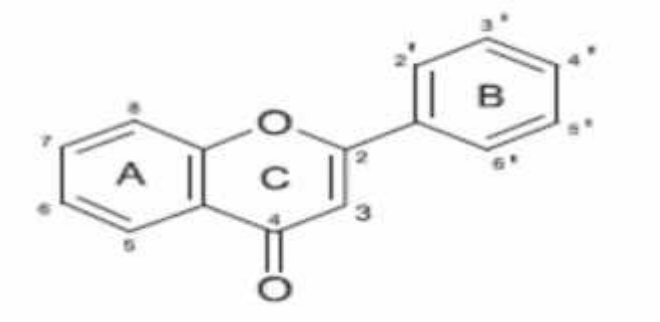


Figure 1. Nuclear structure of flavonoids

Flavonoids containing a hydroxyl group in position C-3 of the C ring are classified as 3-hydroxy flavonoids (flavonols, anthocyanidins, leucoanthocyanidins, and catechins), and those lacking it as 3-desoxyflavonoids (flavanones and flavones). Classification within the 2 families is relied on the differing in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B- rings (Heim et al., 2002). Flavonoids connected to one or more sugar molecules are known as flavonoids glycosides, while others, are called aglycones. With the exception of flavanols (catechins and proanthocyanidins), flavonoids occur in plants and most foods as glycosides (Walle, 2004).

Metabolism of flavonoids in ruminants

Intestinal Microorganisms tract transform and degrade polyphenolic compounds (Martin, 1982). In the rumen fermentation, flavonoid glycosides such as rutin and naringin can be hydrolyzed and their common flavonoid heterocyclic ring subjected to a cleavage process (Lowry and Kennedy, 1996; McSweeney and Mackie, 1996) to produce phenolic acids such as 3, 4-dihydroxyphenylacetic acid from isoquercitrin (Schneider et al., 1999) and quercetin (Winter et al., 1989), or phenylacetic acid (PAA) from naringenin (Winter et al., 1989).

Although, Gladine et al. (2007c) reported no modifications happen to aglycone fraction structure of the flavonoids during the ruminal fermentation due to necessary hydrolysis of the glycosidic fraction before absorption. However, it is likely that the hydrolysis of the glycosidic fraction occurred in the rumen rather than in the intestine.

After absorption, flavonoids are transported to the liver, where they form glucuronide and/or sulfate or methyl conjugates before being excreted through the urine or and faeces (Spencer et al., 2008).

It was reported that polymeric flavanols (proanthocyanidins) are not decomposed into bioavailable monomers in the monogastric digestive system and it was concluded that only the monomeric components of flavanols (catechin and epicatechin) are absorbed in monogastrics (Nakamura and Tonogai, 2003). Contrary to monogastrics, Gladine et al. (2007c) showed that ruminants can benefit from the strong antioxidant properties of polymeric proanthocyanidins by metabolizing them into bioavailable compounds with intact flavonoid-ring structure (epicatechin).

Bioavailability of flavonoids

Many factors such as chemical structure, absorption, its distribution and elimination determine the biological effects of flavonoids (Heim et al., 2002).

Isoflavones and flavonols seems to have higher bioavailability in monogastric compared with the other subclasses of flavonoids while anthocyanins are reported to have the lowest (Hollman, 2004), whereas, Gladine et al. (2007c) describe higher proanthocyanidins bioavailability of in ruminants compared with the other flavonoids subclasses.

Flavanone bioavailability was studied in healthy rats offered 0.5% naringin (Silberberg et al., 2006), the main plasma metabolites of naringin (naringenin glucuronides) were reached 17.3 µM in plasma after 6 hours where the total amount of flavanones excreted in urine represented 4.6 % of the ingested dose.

Impacts of using the flavonoids in the rations*: At the animal's level*

Control and prevention of acidosis

Acidosis is a pathological condition associated with decrease in alkali (base excess) in body fluid relative to acid (hydrogen ion) content (Owens et al., 1998). Rumen lactic acidosis (grain overload, grain poisoning, acute indigestion) develops in sheep and cattle that have ingested large amounts of rations rich in ready fermentable carbohydrates (Crichlow and Chaplin, 1985; Nocek, 1997). The resulting production of large quantities of volatile fatty acids (VFA) and lactic acid decreases rumen pH to non-physiological levels, simultaneously weakening the

buffering capacity of the rumen, and reduces the efficiency of rumen flora and fermentation. Lactic acidosis can cause ruminitis, metabolic acidosis, lameness, hepatic abscessation, pneumonia and death (Lean et al., 2000). This acidotic process could be conventionally differentiated in different pathological forms, as acute and sub-acute.

Acute acidosis occurs with rapid grain overload and may result in the death of the animal, severe illness, liver abscesses, etc. If these problems are prolonged, the low ruminal pH may result in damage to the ruminal wall, reduced absorption capacity, depressed feed intake and subsequent health problems as it is known as sub-acute acidosis.

Production and consumption of lactic acid is a key point in sub-acute acidosis, lactate-fermenting bacteria can rapidly metabolize lactate to VFA, as the pH nears 5.0, below that for a sustained period, the growth of lactate-fermenting bacteria is inhibited, and lactate begins to accumulate. Therefore, sub-acute acidosis has the potential to become lactic acidosis if the pH of 5.0 is sustained for a time (Nagaraja and Titgemeyer, 2007). In this level, suspension of lactic acid production is become a critical point, during the acidosis challenge, *S. bovis* titers increased and the maximum titers coincided with the greatest lactate concentrations.

It is shown that flavonoids (e.g. naringine), degrades to aglycone to form naringein in the rumen (Gladine et al., 2007c) and rumen microflora can break down the aglycone ring into phenylacetic acid, which is an antimicrobial compound and may inhibit the lactic acid producer bacteria (*i.e S. Bovis*, Winter et al. 1989).

Anti-Microbial effect of flavonoids

Flavonoids could exert synergistic antimicrobial effects in the animal's digestive system or in meat when polyphenol administration is combined with low temperatures, low pH, and anaerobic conditions (Ahn et al., 2002). Flavonoids have shown inhibitory effects on Gram-positive as well as Gram-negative bacteria (Gadang et al., 2008) and the mechanisms by which flavonoids can act as antibacterial are summarized in:

1-Inhibition of nucleic acid synthesis

2-Inhibition of cytoplasmic membrane function

3-Inhibition of energy metabolism

Inhibition of nucleic acid synthesis was described by Mori et al. (1987) and a negative effect of flavonoids on bacterial oxygen consumption and energy metabolism was demonstrated by

(Haraguchi et al., 1998). Different mechanisms have been reported to describe the negative effect of flavonoids on membrane function. Ikigai et al. (1993) showed that they may penetrate in the lipid bilayers and disrupting the barrier function or directly cause membrane fusion, cell content leakage and bilayers aggregation (Ikigai et al., 1993).

The main structural features of flavonoids required for efficient antibacterial could be summarized as follows: (1) hydroxyl groups at position 2′, 4 ′ - or 2 ′, 6 ′ of the B ring (2) hydroxyl groups at 5, 7 of the A rings (Tsuchiya et al., 1996).

Flavonoids demonstrated inhibitory properties against certain pathogens including L. monocytogenes, *E. coli* O157:H7, *Salmonella Typhimurium*, *Campylobacter jejuni*, and others including *S. aureus*, *Staphylococcus epidermidis*, *Salmonella enteriditis*, *Shigella flexneri*, *Shigella dysenteriae*, and *Vibrio cholera* (Gadang et al., 2008; Hamilton-Miller, 1995; Toda et al., 1991) also by adding a natural flavonoid source as citrus fiber into the mortadella preparation it reduces aerobic and lactic acid bacteria counts (Viuda-Martos et al., 2010).

Flavonoids can act as both, indirectly (like ionophores) 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 to the expense of acetate and butyrate (Chen and Wolin, 1979), and also the may be toxic to methanogens.

Antioxidant effect of flavonoids

The efficiency of vitamin E as an antioxidant is limited when animals are fed diets rich in polyunsaturated fatty acid such as *n−3* PUFA (Allard et al., 1997). Moreover, it has a pro-oxidant action when high doses are ingested, or in the absence of other antioxidants able to recycle the oxidized form of vitamin E (Mukai et al., 1993). Hence, possibility of supplementing cattle diets with natural antioxidant compounds, alternative to vitamin E, as a means of improving beef quality requires investigation.

Prior and Cao (2000) reported that the antioxidant capacities of many flavonoids are much stronger than those of vitamins C and E and, hence, supplementing ruminants with these compounds may be useful particularly when the animals fed diet rich in polyunsaturated fatty acids such as *n*-3 fatty acids which are highly susceptible to peroxidation in plasma and tissues (Gladine et al., 2007a; Gladine et al., 2007b; Gladine et al., 2007c).

Mode of action

The mechanisms by which the flavonoids can act as antioxidant are based on:

1- Direct scavenging of reactive oxygen species (Pietta, 2000)

2- Activation of antioxidant enzymes (Nijveldt et al., 2001)

3- Metal chelating activity (Ferrali et al., 1997)

4- Reduction of α-tocopheryl radicals (Heim et al., 2002)

It has been reported that flavonoids are able to scavenge free radicals directly by hydrogen atom donation. Radicals are made inactive according to the following equation, where R• is a free radical and Fl-O• is a flavonoid phenoxyl radical (Figure 2; Procházková et al. (2011).

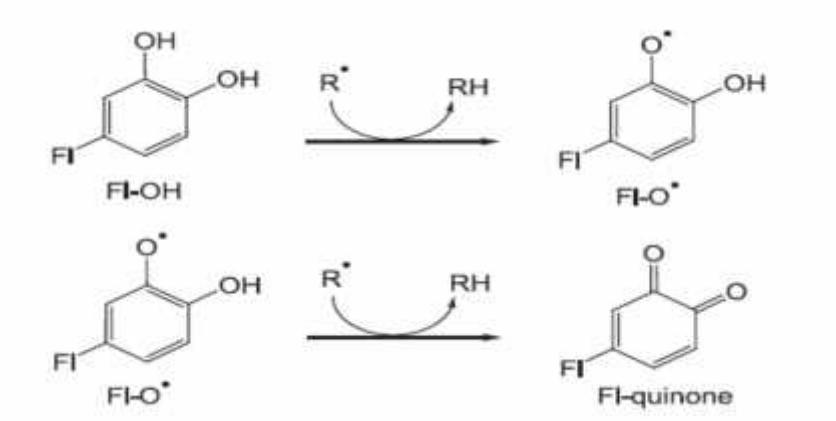


Figure 2: Scavenging of reactive oxygen species (R•) by flavonoids. The free radical Fl-O• may react with a second radical, acquiring a stable quinone structure.

The main structural features of flavonoids required for efficient radical scavenging could be summarized as follows (Croft, 1998): (1) an ortho-dihydroxy (catechol) structure in the B ring, for electron delocalization (2) 2, 3-double bond in conjugation with a 4-oxo function in the C ring provides electron delocalization from the B ring (3) hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the oxo group.

According to the previously stated criteria, flavonols quercetin and myricetin should be the most effective radical scavengers in the aqueous phase (Rice-Evans et al., 1996).

There are differences in antioxidant activity between all the chemical compounds belong to the flavonoids group which might be attributed to the variety of substituents on the flavonoid molecules. Rice-Evans et al. (1996) compared radical scavenging activities of flavonoids and phenolic acids occurring in higher plants by using the Trolox (a water-soluble α tocopherol

analog) - equivalent antioxidant capacity (TEAC) assay. They showed that all phenolic acids demonstrated lower TEAC values than flavonoids. The quercetin metabolite 3,4-dihydroxyphenylacetic acid (TEAC = 2.19) was substantially weaker than quercetin (TEAC = 4.7) but remained over twice as effective as vitamin E. Regarding dietary flavonone, citrus flavanone naringenin, also possess some antioxidant activities, although this activity is poorer compared with many other polyphenols (TEAC = 0.24 and 1.53 for naringin and naringenin, respectively; Rice-Evans et al. 1996).

Specific flavonoids are known to chelate iron, thereby removing a causal factor for the development of free radicals. The *O*-dihydroxyphenyl groups of flavonoids are excellent chelators of iron (Santos-Buelga and Scalbert, 2000) which is considered a catalyst for initiation of lipid oxidation and generation of hydrogen peroxide.

Flavonoids can act as hydrogen donors to α-tocopheryl radical, which is a potential prooxidant. As well, by interaction with α-tocopheryl radical, they possess a great potential to delay the oxidation of LDL. Flavonoids (kaempferol, morin, myricetin and quercetin) showed a varying protective activity against depletion of *α*-tocopherol in LDL, with kaempferol and morin being less effective than myricetin and quercetin (Zhu et al., 1999). Catechins may be even more effective than ascorbate in regenerating *α*-tocopherol in micellar solution (Mukai et al., 2005). Naringin also reported to spare or recycle vitamin E (Jeon et al., 2002; Jeon et al., 2001).

The efficiency of flavonoids to reduce lipoperoxidation depends mainly on the type of tissue. Gladine et al. (2007b) studied the effect of flavonoids on lipoperoxidation intensity malondialdehyde production (MDA) in different tissues and showed that MDA production was reduced in rats' liver given flavonoids, whereas in *Longissimus thoracis* tissues, the reduction MDA production was much less pronounced and irrelevant. The authors suggested that liver metabolism of flavonoids disturb their activity.

Mechanism of action in meat systems

In membrane model system studies, antioxidant efficiency of flavonoids has been found to be dependent not only on their redox properties but also on their ability to interact with membranes (Pazos et al., 2006; Saija et al., 1995). In this sense Movileanu et al. (2000) reported the ability of flavonoids to penetrate the lipid membranes and Van Dijk et al. (2000) showed that flavonoids hydrophobicity decreases as OH groups increased. Thus, the polar flavonoids, (low OH groups),

which are located deeper in the membrane and have the ability to accumulate increasing polarity due to an increasing number of hydroxyl groups that provides the flavonoids higher propensity towards the lipid/ water interface.

Lee et al. (2006) reported that that the most lipophilic components in cranberry diet (such as flavonols) were the most effective in inhibiting lipid oxidation in mechanically separated turkey and cooked pork compared to the more water-soluble proanthocyanidins and anthocyanins.

In meat systems, flavonoids demonstrate antioxidant activity by reducing the amount of primary lipid oxidation products (e.g. lipid hydroperoxides and hexanal) and secondary lipid oxidation products (e.g. thiobarbituric acid reactive substances-TBARS) in beef, chicken, and turkey during refrigerated storage (Ahn et al., 2002; Brannan and Mah, 2007).

Prooxidant action of flavonoids

Flavonoids can act, under certain condition, as prooxidants and, hence, promote the oxidation of other compounds. Prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups in a flavonoid molecule (Cao et al., 1997). Mono- and dihydroxyflavonoids demonstrated no detectable prooxidant activity, while multiple hydroxyl groups, especially in the B-ring, significantly increased production of hydroxyl radicals in the Fenton reaction (Heim et al., 2002).

It is now well accepted that certain flavonoids have been reported for their prooxidant nature reducing transition of metal ions, i.e iron. It is presumed that these flavonoids can exert prooxidant effect by promoting Fenton or Haber–Weiss reaction (Galati and O'Brien, 2004). Flavonoids including myricetin and quercetin possess a high Fe^{3+} reducing activity, and hence exert higher prooxidant effect providing evidence for the presence of the catechol group in the B-ring , the 3-hydroxyl group in C-ring and 2,3-double bond in conjugation with the 4-oxo group in the C-ring (Silva et al., 2002). The flavonoids that lack the catechol group in the B-ring, 3-hydroxyl group in C-ring and also 2, 3-double bond, i.e. naringin, may not have such prooxidant activity (Jagetia and Reddy, 2011).

Impacts of using the flavonoids in the rations: At the environment level

Mitigating methane production

Methane is a potent greenhouse gas (GHG) which contributes to global warming and is 25 times more powerful than CO_2 (Solomon et al., 2012). Global methane production is about 535 MT, in which 160 MT is generated naturally and 375 MT is derived from anthropogenic activity (Houghton et al., 1996). It is expected this amount will increase by 28% in 2030 reaching to 480 MT (Höglund-Isaksson, 2012). The livestock is responsible for a significant amount (up to 23 %) of the anthropogenic emissions of the methane at the global level (Khalil, 2000) and in Spain this contribution could exceed over 35% (Cambra-López et al., 2008). Rumen and large intestine in mono-gastric animals are the organ in which the fermentation occurs, that results in the production of short chain fatty acids (VFA; acetic, propionic and butyric acid), urea (NH_3, Cao et al. 2012), heat (Jørgensen et al., 2011) and gases (carbon dioxide: CO_2; hydrogen: H_2 and methane: CH_4), however, due to the fermentation of the slurry from the pit and storage pool, the production of these gases are continuous (Steinfeld et al., 2006). Spain as an agricultural base country has around 5.9 million head of cattle (MARM, 2010) and responsible for production of 84 kg CH_4 animal/year in dairy cattle or 107 kg CH_4 animal/year in fattening calves. Methane production, naturally prevents the accumulation of the dihydrogen end product released by bacteria, protozoa and fungi during the fermentation of the carbohydrate fraction (Morgavi et al., 2010), it is produced as the major sink of hydrogen released from volatile fatty acids (VFA) synthesis and negatively correlated with energy utilization and microbial mass synthesis on ruminants (Orskov et al., 1968; Yáñez-Ruiz et al., 2010), and its emission is a potential contributor to the global warming phenomenon (Johnson et al., 2002).

Mode of action

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 with carbon or electron flow at a point upstream in the microbial food chain. In this approach, hydrogen would not accumulate and propionate would increase at the expense of acetate and butyrate. 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). In

polyphenolic compounds such as flavonoids and anthraquinone-derivatives, it is demostrated that methane inhibition occurs at the point of methane formation from hydrogen and carbon dioxide or acetate by methanogenic archaea, and not at the point of hydrogen or acetate formation by fermentative organisms (Odom, 1997). Thus, other hydrogen sinks, *i.e.* an increase of the butyrate and propionate proportions, are promoted, (Garcia-Lopez et al., 1996).

CHAPTER 3

OBJECTIVES AND EXPERIMENTAL DESIGN

The general objective of the presented work was to study on the impacts of a commercial citrus extract rich in flavonoids, Bioflavex® as a regulatory factor for rumen fermentation and animal metabolism.

This general objective was split out in several partial objectives or tasks:

1) To determine "*in vivo*" the effect of the citrus extracts rich in flavonoids, Bioflavex®, on rumen fermentation characteristics and analyzing its impact over specific ruminal bacteria involved in lactate production under experimental acidotic induction.

2) To study "*in vivo*" the effect of Bioflavex® supplementation on animal's performance and rumen fermentation in finishing heifers fed high-concentrate diets.

3) To analyze the effect the flavonoids mixture and its pure ingredients on methane production and methanogenic species under *in vitro* condition using rumen liquid from growing steers fed high concentrate diets.

4) To determine *in vivo* the effect of different dietary doses of Bioflavex® in finishing Friesian bulls fed high concentrate diets on growth, carcasses conformation and antioxidant capacity to prevent lipid oxidation in meat.

To achieve the previous defined objectives several experiment were planed:

In the **first** experiment, the effect of Bioflavex® on the development of rumen acidosis was evaluated. Eight Holstein-Friesian cross-breed heifers were fitted with rumen cannula and kept indoors. Animals fed grass hay at a maintenance level. The experiment was conducted using a crossover (2 x 2) design that had two treatments and two periods (22 days each). On day 21 of the experiment, the animals were fasted and on day 22, rumen acidosis was induced by applying, manually, 5 kg of ground wheat through the cannula. Half of the animals received Bioflavex®, which was mixed within the ground wheat, and the other half did not receive Bioflavex® (known as CTR). From day 19 to 22 of each experimental period, rumen pH was measured every 22 min and the pH values were recorded and stored automatically. Samples of rumen contents were

collected before (0 h) and 2, 4, 8, and 24 h after heifers were given the ground wheat supplement and analyzed for lactate, NH_3-N, VFA and microbial DNA analyses. After 15 d as transition period, the same 22 d experiment was repeated.

Following the first experiment, and to evaluate the capacity of aforementioned commercial blend to improve rumen fermentation characteristics and performance of cattles in feedlot, the **second** experiment using forty-eight Fleckvieh heifers was designed. Selected 48 heifers were assigned to one of 4 blocks (12 heifers/ block), and each block was divided into two pens and randomly, one pen of each block was assigned to the concentrate without (CTR) or with Bioflavex® and barley straw which were offered *ad libitum*. Straw and concentrate DMI were calculated weekly as well. Then, to confirm the obtained results from the first experiment, two heifers from each pen (16 animals) were selected randomly and fitted with a permanent rumen plastic cannula into their dorsal rumen sac to be sampled for pH values, NH_3-N and VFA concentrations. Urine spot samples were also collected from the cannulated heifers by vulva massage to quantify the concentrations of purine derivatives (PD: allantoin, uric acid) and creatinine in order to follow the duodenal absorption of purine bases (PB) and rumen microbial outflow.

The **third/four** experiments were designed to confirm in a more controlled assays the impact and extension of flavonoids supplementation on rumen fermentation (Ruminal pH, lactate, NH_3-N and VFA concentrations together with the related microbiota abundance) simultaneously under both, *in vivo* and *in vivo* conditions. In the *in vivo* trial, eight crossed, 9 month old Holstein-Frisian calves fitted with a cannula in the dorsal sac of the rumen were used. Animals were housed in individual pens and randomly assigned to each of two experimental treatments; concentrate supplemented with or without the Bioflavex® plus barley straw for 24 days in a 2×4 cross over design, with two periods of 12 days and four calves per period and treatment. Concentrate and straw were supplied *ad libitum* in the separated feeders, and consumptions and orts were daily recorded on DM basis. Calves were weighed individually at the onset and the end of each experimental period. The impact of Bioflavex® versus its pure flavonoid components on ruminal fermentation processes and also rate and characteristics of fermentation were studied by the mean of four *in vitro* incubations series using rumen liquor from four rumen cannulated growing steers fed high concentrate diet at *ad libitum* for at least 4 weeks. Gas production and its liberation rhythm were calculated; and also the effect of

Bioflavex® and its main components on evolution of pH, lactate, NH_3-N and VFA concentration plus related microbiota abundance were revealed *in vitro*.

The **fifth** trial was planned to study the anti-microbial activity of the flavonoids mixture over rumen methanogen population and methane emission, analyzing the effect of the Bioflavex® mixture and in camprasion with its pure flavonoid ingredients. Four batches or incubation series were conducted using rumen liquor from four rumen cannulated growing steers fed commercial concentrate and barley straw *ad libitum*. Gas and methane volume and their production rate at each incubation time were calculated, besides, the pH was determined and the ammonia nitrogen and VFA concentration were measured. Related abundance of methanogenic archaea responsible for methane production and the abundances of certain types of ruminal bacteria related to the fermentation process were studied as well.

CHAPTER 4

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

The context of this chapter is published as a research article in Journal of Animal Science (Volume 90, Pages 4975-4984) with DOI number 10.2527/jas2011-4955.

ABSTRACT

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

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

INTRODUCTION

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

Bioflavex® (**FL**; Exquim S.A., Barcelona, Spain) is a blend of natural flavonoid extracts comprising mostly naringine (200 g/kg), which is extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradisi*; 400 g/kg). From the existing literature it was hypothesized that Bioflavex® could exert some antimicrobial activities and the objective of this study was to verify the Bioflavex® effects on ruminal pH and specific ruminal bacteria involved in lactate production under 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 experimental station (Prat de Llobregat, Spain). Animals were fed grass hay (11.2% CP, 62.2% NDF, and 34.4% ADF on a DM basis) at a maintenance level (7.2 kg DM/d). The experiment was conducted using a crossover (2 × 2) design that had 2 treatments and 2 periods (22 d each). On d 21 of the experiment, the animals were fasted and on d 22, at 0800 h, rumen acidosis was induced by applying, manually, 5 kg of ground wheat (11.2% CP, 11.0% NDF, and 3.7% ADF on a DM basis) through the cannula. Half of the animals received FL (300 mg/kg), which was mixed in with the ground wheat, and the other half did not receive FL (Control: **CTR**). After a 15-d transition period, the same 22-d experiment was repeated.

Experiment 2.

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

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

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

Measurements and Sample Collection

Experiment 1.

From d 19 to 22 of each experimental period, rumen pH was measured every 22 min using a pH meter (X-Mate Pro MX 300, Mettler-Toledo, Barcelona, Spain) that was capable of recording and storing pH values automatically (for details, see Bach et al., 2007). Samples of rumen

contents were collected before (0 h) and 2, 4, 8, and 24 h after heifers were given the ground wheat supplement, which were stored frozen (–20° C) until they were analyzed for lactate, 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 mercuric chloride, 20 mL/L orthophosphoric acid, and 2 g/L 4-methylvaleric acid (internal standard) in distilled water and frozen until the VFA analysis. For the microbial DNA analyses, 50-mL rumen samples were centrifuged at 6500 × *g* for 15 min at 4 °C. The supernatant was discarded and the homogenized pellet was distributed among 0.25 g aliquots that were stored frozen at –80°C until they were analyzed.

Experiment 2.

Animal BW and the consumption of concentrate and straw were recorded weekly. After d 49 of the experiment, rumen fermentation was characterized once a week at 0800 h, 1000 h, and 1200 h. After weighing the animals at 0800 h, the rumen contents (about 200 mL) from the cannulated animals were collected using a vacuum pump, pH was recorded, and the rumen samples were filtered through 2 layers of cheesecloth. Two subsamples were used to quantify NH_3–N and VFA concentrations 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 °C until they were used to quantify the concentrations of purine derivatives (**PD**; allantoin and uric acid) and creatinine. The urinary PD:creatinine ratio was used as an index of the duodenal absorption of purine bases (**PB**) and rumen microbial outflow, under the assumption that PB are appropriate microbial markers (Pérez et al., 1997).

Chemical and Microbial Analyses

After DM determination using an oven at 60° 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 °C for 4 h), CP, and ether extract, following the Association of Official Analytical Chemists (AOAC, 1990). Dietary NDF and ADF concentrations were measured following the method of Van Soest et al. (1991) using sodium sulfite and α-amylase. To measure rumen 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). Rumen VFA concentrations were measured based on Jouany (1982) method. L+ plus D-lactate was determined by the colorimetric method proposed by Taylor (1996). Urinary concentrations of

allantoin, uric acid, and creatinine were measured using the HPLC method described by Balcells et al. (1992). Total microbial DNA was extracted using the repeated bead beating and column (**RBB+C**) method (Yu and Morrison, 2004) using bead beating in the presence of high concentrations of SDS, salt, and EDTA, and subsequent DNA purifications were performed using QIAamp columns from the Stool DNA Kit (QIAGEN, Valencia, CA).

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

Calculations and Statistical Analyses

Urinary PD: creatinine ratios were measured in the spot samples that were collected from the cannulated heifers. Absolute values of PD (mmol/d) were calculated 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 effect. All of the animals were weighed at the beginning of the experiment and thereafter once a week until the end of the experiment, and the data were used to calculate the ADG as the slope of the linear regression of BW against time. For the rumen and urine data, the effect of time after feeding (hours) and interactions were considered as fixed effects. In the model, pen (n = 8) was the experimental unit for all of the statistical analyses. Significant differences and tendencies were declared at $P < 0.05$ and $P < 0.10$, respectively.

RESULTS

Experiment 1

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

Changes in the rumen pH during the 72-h period are shown in Figure 1. The interaction between treatment and feeding phase (i.e., maintenance, fasting, and acidosis induction) on rumen pH was significant ($P < 0.01$). After the ground wheat was administered, rumen pH decreased, more so in the CTR (5.98; SEM = 0.029) than in the FL heifers (6.29; SEM = 0.031). Mean values of ruminal NH_3–N and VFA concentrations and relative VFA proportions are presented in Table 3. Treatment did not affect total VFA concentration or molar proportions of propionate in the rumen. As expected, and paralleling the changes in rumen pH, total rumen VFA concentrations and molar proportions of propionate increased at 2 and 4 h after ground wheat was administered through a rumen cannula; however, at 8 h, concentrations declined. Compared with the CTR heifers, the FL heifers had a decreased rumen molar proportion of acetate ($P < 0.05$) and tended to have reduced concentrations of butyrate ($P = 0.06$) and acetate to propionate ratio ($P = 0.09$). The posttreatment changes in rumen acetate and butyrate (mol/100 mol) were similar to those that were observed in VFA concentrations.

Rumen lactate concentrations, lactate-producing bacteria (*S. bovis*), and lactate-consuming bacteria (*S. ruminantium* and *M. elsdenii*) titers are presented in Table 4. Data correspond to samples that were taken at time 0 (before acidosis onset) and after acidosis induction (the samples from 4 and 8 h after acidosis induction were pooled together, 50:50). Rumen lactate concentrations increased ($P < 0.001$) after the wheat supplement was administered, and the

increase in rumen lactate concentrations between 0 h and 4 to 8 h after wheat supplementation was numerically less ($P = 0.09$) in the FL heifers (0.41 to 1.18 mmol/L) than in the CTR heifers (0.41 to 1.54 mmol/L; SEM = 0.12). The corresponding titres of *S. bovis* and *S. ruminantium* increased ($P < 0.05$) in the FL and CTR groups after wheat supplementation, and *M. elsdenii* titers tended ($P = 0.09$) to increase in the FL heifers. Heifers supplemented with FL had greater ($P < 0.05$) *M. elsdenii* titres than CTR heifers.

Experiment 2

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

Average pH (6.42 vs. 6.09; SEM = 0.03) and VFA concentrations (74.8 vs. 65.7 m*M*; SEM = 1.86) in cannulated 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 administration. Ammonia-N concentrations were less in the FL than in the CTR heifers [10.2 vs. 41.5 mg/L (SEM = 2.81), respectively].

Rumen molar proportions of acetate (53.9 vs. 59.4 mol/100 mol; SEM = 0.60) were less ($P < 0.01$) and proportions of propionate (35.5 vs. 28.1 mol/100 mol; SEM = 0.57) were greater ($P < 0.01$) in the FL than in the CTR heifers; consequently, the acetate-to-propionate ratio was greater ($P < 0.01$) in the CTR heifers than in the FL heifers [2.34 vs. 1.65 (SEM = 0.06), respectively]. 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 performance can be diminished (Owens et al., 1998). Chronic and acute acidosis are indicated by rumen pH values of

5.6 and 5.2, respectively (Cooper and Klopfenstein, 1996). In Exp. 1, in which a wheat supplement was used to induce acidosis, rumen pH was reduced from 6.70 [SEM = 0.22; which is within the normal range for roughage diets (France and Siddons, 1993)] to 5.22 (SEM = 0.19), and the average amount of time in which pH was <5.5 and <6.0 was 0.5 and 4 h, respectively. The minimum pH values (5.0 to 5.5) registered were within the pH ranges reported in studies that used grain engorgement to induce subacute acidosis in (Bauer et al., 1995; Krehbiel et al., 1995; Goad et al., 1998).

During acute acidosis induction (pH 3.9 to 4.5; Nagaraja et al., 1985), lactate concentrations (mmol/L) may exceeded 50; however, under subacute acidosis conditions lower increases in lactate concentrations has been also reported [e.g., <10 (Burrin and Britton, 1986), 5 (Goad et al., 1998), or virtually no increase at all; e.g., <0.4 (Coe et al., 1999; Bevans et al., 2005)]. Moreover in our case, the lack of synchronization between the lowest pH records (9 to 12 h after wheat supply) and lactate sampling (pooled samples harvested at 4 and 8 h) could mask the negative relationship between pH and lactate. Lactic acid accumulates when the amount of rapidly degradable carbohydrates increases suddenly in the rumen, which stimulates the proliferation of rapidly growing lactic acid-producing bacteria, for example, *S. bovis*, to the point where the growth of lactic-producing microorganisms exceeds the growth rate of lactic acid-using bacteria (Russell and Hino, 1985); consequently, lactic acid accumulates. In our experiment, during the acidosis challenge, *S. bovis* titers increased and the maximum titers coincided with the greatest lactate concentrations; however, it is likely that the simultaneous increase in lactate-consuming bacteria (*S. ruminantium* and *M. elsdenii*) might have mitigated the production of lactic acid. After acidosis has been induced (during an adaptation to a high-concentrate diet), there can be a short-term increase in lactate-consuming bacteria (Goad et al., 1998; Tajima et al., 2001) although it has been demonstrated that most lactate-consuming bacteria cannot tolerate low pH conditions (Russell and Hino, 1985). The activity of flavonoids can have an effect on the microbial growth of pathogenic (Wu et al., 2009) and nonpathogenic (Broudiscou and Lassalas, 2000) bacteria. Moreover, in the human intestine the flavonoids genistein and daidzein has been demonstrated to be able to modify the predominant microbiota (Schoefer et al., 2002; Clavel et al., 2005). Naringin, the main component of Bioflavex®, degrades to aglycone (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 antimicrobial

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 rumen ecosystem adapted to the presence of polyphenol compounds.

The addition of the commercial FL mixture was able to partially buffer rumen acidification although this effect was not consistently supported by recorded values in rumen VFA concentration. Discrepancies between rumen VFA concentrations and pH have been observed in both experiments. In the first experiment, large differences in rumen pH between treatments were observed whereas no differences in rumen VFA concentrations between treatments were observed, and in the second experiment FL heifers had greater VFA concentrations and greater rumen pH compared with CTR heifers. Changes in rumen pH are primarily determined by fermentation products, VFA and lactic acid, derived from carbohydrate fermentation (Owens et al., 1998). However, as Sauvant et al. (1999) described, VFA concentration only explained 32% of the variation of the rumen pH observed. Any change in rumen pH is buffered by feed ingested, saliva, and dietary buffers added, and rumen pH is also influenced by rumen rate of passage. In Exp. 1, the lowest pH values were recorded 9 to 12 h after wheat supply and rumen VFA and lactate concentrations were measured latest at 8 h after wheat supply. The lack of VFA and lactate data when the pH records reached the lowest value, avoid to confirm a direct relationship between VFA or lactate concentration (or both) on rumen pH; however, the numerical less lactate concentration in CTR than in FL heifers at 8 h after wheat supply may indicate that FL supplementation prevented heifers from lactic acidosis. Nagaraja and Titgemeyer (2007) indicated that in subacute acidosis, the reason for pH to decline below 5.6 is accumulation of VFA, which is a combination of overproduction (increased substrate) and possibly decreased absorption. Although lactic acid is produced during subacute acidosis, it does not accumulate because lactate-fermenting bacteria remain active and rapidly metabolize it to VFA However, as the pH nears 5.0 or below for a sustained period, the growth of lactate-

fermenting bacteria is inhibited, and hence lactate begins to accumulate. Therefore, subacute acidosis has the potential to become lactic acidosis if the pH of 5.0 is sustained for a time (Nagaraja and Titgemeyer, 2007). In Exp. 2, heifers supplemented FL consumed more straw than CTR heifers; this may stimulate rumination and saliva production buffering rumen pH, explaining the greater pH observed in FL compared with CTR heifers even if rumen VFA concentration was greater in FL than in CTR heifers. As mentioned previously, correlation between rumen VFA concentration and pH is low, and buffering mechanisms such as saliva have a great impact on rumen pH regulation. Mechanisms whereby flavonoid supplementation may stimulate straw consumption are unknown. So, flavonoids addition seems to be effective in preventing pH reduction through modifying the activity of lactating-consuming bacteria but also may have a direct buffer effect or stimulating straw consumption or both. In both of our experiments, flavonoid supplements increased rumen molar proportions of propionate and reduced the acetate-to-propionate ratio, which suggests that flavonoid supplements might have altered the entire rumen microbiota and stimulated the growth of propionate-producing bacteria. Furthermore, FL supplements reduced rumen NH_3–N concentrations and increased urinary PD excretion (an index of the duodenal flow of microbial N). The reduction in rumen ammonia concentrations coupled with a significant increase in the duodenal flow of microbial N suggests an improvement in rumen N use.

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

In our study, the slow ADG and the increased residual variation (CV = 9.37%) might have masked the effects of the flavonoid mixture. The heifers consumed large amounts of a corn-based concentrate; however, the CTR heifers did not exhibit rumen pH values that are indicative of subclinical acidosis (Cooper and Klopfenstein, 1996). It was true that FL heifers consumed more straw than the CTR animals, and the regulatory effect of saliva might have altered the pH; however, the small difference (<2% of total DMI) between the 2 groups suggests that any effect of differences in straw intake were negligible. Allowing adequate time for animals to adapt to a

high-concentrate diet, the low degradability of corn starch (Owens et al., 1998) and a proper feeding space ratio (Devant et al., 2007; González et al., 2009) reduce the likelihood of acidosis, which would forestall the expression of any effects of flavonoids on the impaired rumen fermentation.

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

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

In summary, flavonoid supplementation might be effective in improving rumen fermentation and reducing the incidence of rumen acidosis. This effect of flavonoids may be partially explained by increasing the numbers of lactate-consuming microorganisms (e.g., *M. 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.

Footnotes

This research was financed by Exquim S.A, San Cugat, Barcelona, and the TRACER project (Ref: PET2008-0116). The authors thank the staff of the IRTA (Experimental station, Prat de Llobregat, Barcelona) and the Chemistry Departments of the IRTA and UAB for their assistance.

Table 1. Ingredients and nutrient composition of the concentrate fed to experimental heifers (Experiment 1)

Ingredients[1] (%):	CTR	FL
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)		
CP	132	138
EE	58.0	62.0
NDF[4]	270	256
Ash	68.0	63.0

[1]CRT = control, FL = supplemented with a commercial flavonoid mixture (Bioflavex®; 300 mg/ kg, DM ground wheat)

[2]Declared composition of the Vitamin-Mineral Premix [IU/kg]: Vitamin A, 200.000; Vitamin D3, 60.000; [mg/kg]: Vitamin E, 1300; Vitamin B1, 125; Vitamin B2, 550; Vitamin B12, Zinc oxide, 10; Sodium Selenate, 60; Cobalt carbonate, 60 [g/kg]: Nicotinic acid, 2; Magnesium oxide, 9; Copper Carbonate, 7.35; Copper sulphate, 2.5.

[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 RT-PCR amplification cycles for bacteria quantification used in Experiment 1

Microorganism	*16SrRNA* Primers	Reference	RT-PCR amplification cycle
Megasphaera elsdenii	Fw5'GACCGAAACTGCGATGCTAGA 3' Rv5'CGCCTCAGCGTCAGTTGTC 3'	(Ouwerkerk et al., 2002)	1 x (95ºC 10:00min) 45 x (95ºC 00:15min, 57ºC 00:10min, 72ºC 01:00min)
Selenomonas ruminantium	Fw5'TGCTAATACCGAATGTTG 3' Rv5'TCCTGCACTCAAGAAAGA 3'	(Tajima et al., 2001)	1 x (95ºC 10:00min) 45 x (95ºC 00:15min, 53ºC 00:10min, 72ºC 01:00min)
Streptococcus bovis	Fw5'CTAATACCGCATAACAGCAT 3' Rv5'AGAAACTTCCTATCTCTAGG 3'	(Tajima et al., 2001)	1 x (95ºC 10:00min) 45 x (95ºC 00:15min, 57ºC 00:10min, 72ºC 01:00min)

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

	Treatment1			Time [2]						*P*-Value[4]	
Item	CTR (n=8)	FL (n=8)	SEM[3]	0	2	4	8	24	SEM[3]	Tr	H
NH3-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	0.54	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] CRT = control, FL = diet supplemented with a commercial flavonoid mixture (Bioflavex®; 300 mg/ kg DM ground wheat)

[2] Time (h) since a wheat supplement was administered through a rumen cannula

[3] SEM = standard error of the mean

[4] Tr = treatment effect, H = Time since a wheat supplement was administered through a rumen cannula

Table 4. Effects of FL before (0 h) and after onset (4+8) of induced acidosis on lactate concentrations (mg/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[3]	Tr	H	Tr x H
Lactate Concentration (mg/L)									
	87.4	71.7	7.77	36.6	122	8.03	0.44	< 0.01	0.09
Absolute Quantification[5]									
S. ruminantium	53141	57499	8374	39652	70988	8679	0.72	0.02	0.72
S. bovis	42677	31183	9974	20207	53564	9654	0.39	0.02	0.31
Relative Quantification[6]									
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] CRT = control, FL = supplemented with a commercial flavonoid mixture (Bioflavex®; 300 mg/ kg, DM ground wheat)

[2] Time (h) since a wheat supplement; rumen was sampled after 2 h, 4 h, and 8 h, but the last two samples were (50/50) pooled.

[3] SEM: standard error of the mean

[4] Tr = treatment effect, H = hour after feeding effect, T x H = treatment by hour post-feeding interaction

[5] Measurements Units (gene molecules 16S-rRNA/12,5ng of microbial DNA)

[6] Measurement Units ($2^{-\Delta C\,(t)}$)

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

Item	Treatment[1]		SEM[2]	*P*-value[3]		
	CTR (n=8)	FL (n=8)		Tr	W[4]	Tr x W
BW						
Initial kg	325	320	3.25	0.16	-	-
Final kg	402	401	5.25	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
Concentrate efficiency[5],	6.03	5.33	0.15	0.17	0.83	0.74

[1] CRT = control, FL = supplemented with a commercial flavonoid mixture (Bioflavex®; 300 mg/ kg DM ground wheat)

[2] SEM: standard error of the mean

[3] Tr = treatment effect, W = week effect, Tr x W = treatment by week effect

[4] Time corresponds to animal's body weight and feed intake, which were recorded weekly

[5] Concentrate efficiency: Concentrate Intake: ADG (Kg/Kg)

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

Item	Treatment[1]			Hours[2]				Day of experiment[3]					*P*-value[5]				
	CTR (n=8)	FL (n=8)	SEM	0	2	4	SEM	49	56	63	70	SEM[4]	Tr	H	D	Tr x D	Tr x 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[6]																	
PD , mmol/d	101	121	3.24					105	107	113	120	4.58	0.02	-	0.05	0.41	-
PD/C (mol/mol)	1.20	1.40	0.03					1.20	1.30	1.30	1.30	0.05	0.04	-	0.33	0.30	-

[1] Treatment effect: CRT = control, FL = supplemented with a commercial flavonoid mixture (Bioflavex®; 300 mg/ kg, DM concentrate)

[2] Hours after concentrate administration.

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

[4] SEM: standard error of the mean

[5] Tr = treatment effect, H = hour after feeding effect, D = day of experiment effect, Tr x D = treatment by day of experiment interaction, Tr x H = treatment by hour post-feeding interaction

[6] Urinary excretion of purine derivatives (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 excreted (mol/mol). Average creatinine excretion (896 mmol/ kg $BW^{0.75}$) was described by Martín-Orúe et al. (2000).

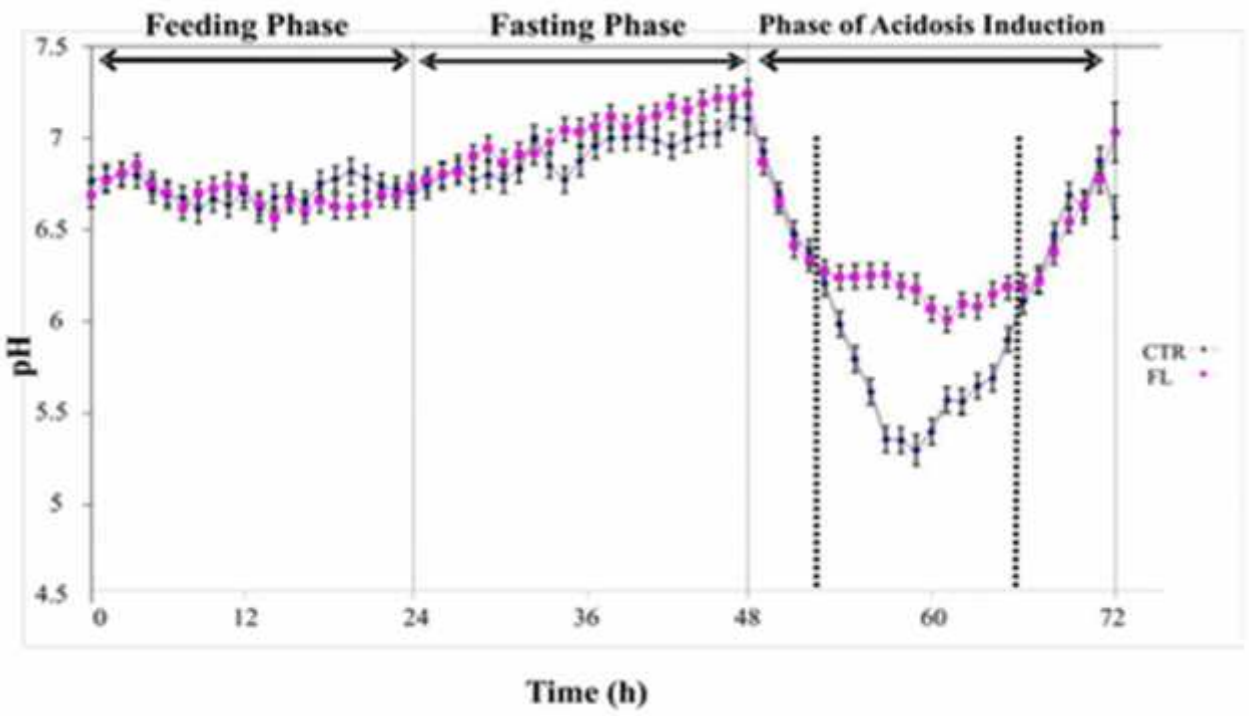
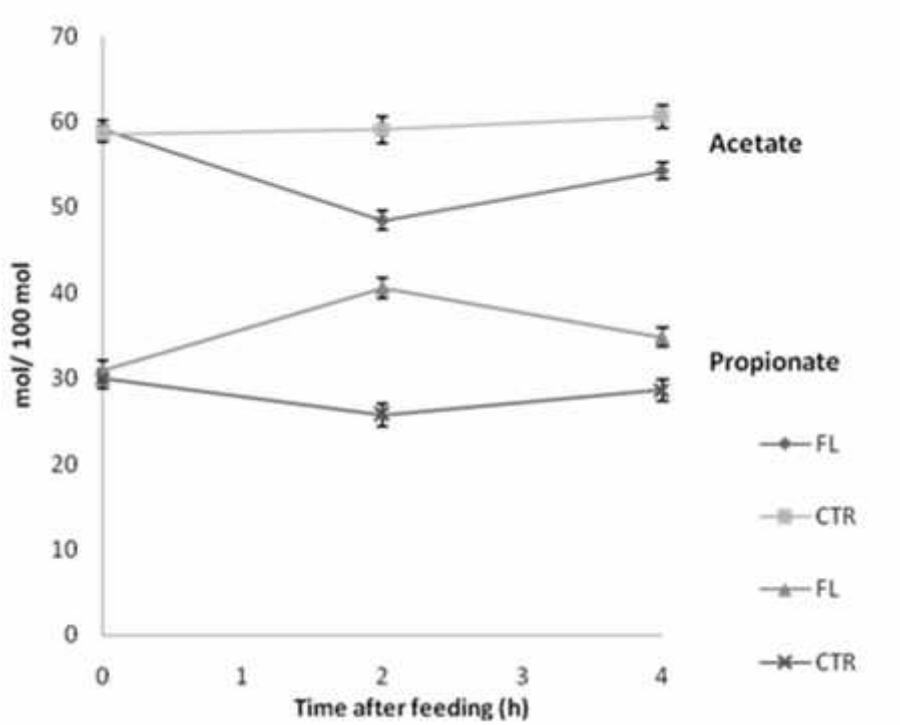
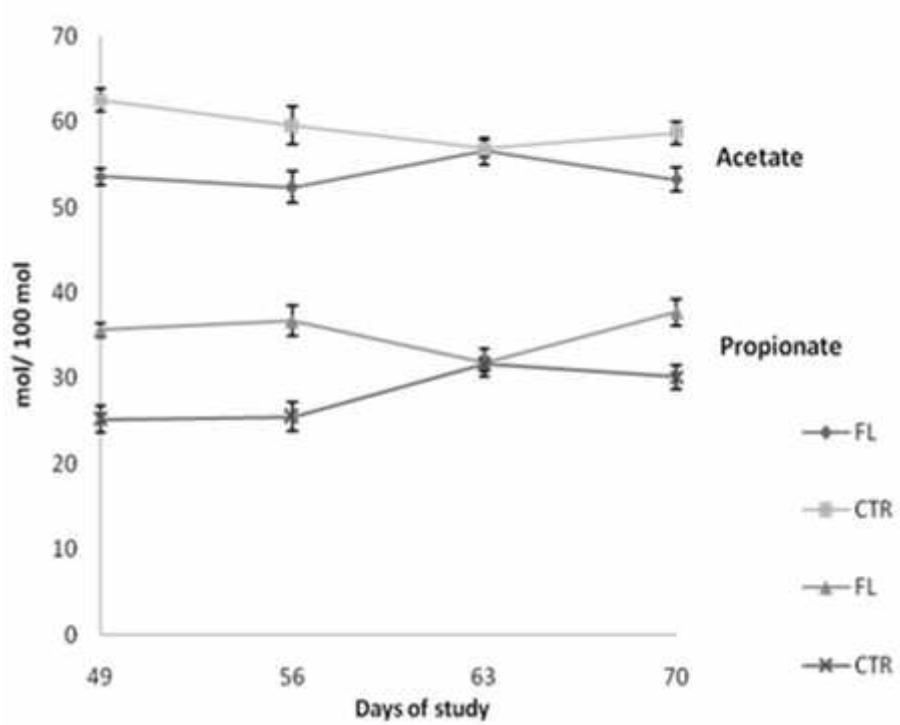

Feeding Phase
Fasting Phase
Phase of Acidosis Induction
7.5
7
6.5
6
5.5
5
4.5
pH
0
12
24
36
48
60
72
Time (h)
CTR
FL

Figure 1: Rumen pH over time (h) where Holstein crossbreed heifers were fed, i) at a maintenance level [Feeding phase] ii) a day before acidosis was induced [Fasting phase, 24h] and, iii) onset of acidosis induction. Animals were supplied with (FL) or without (CTR) flavonoids, and the dotted lines indicate the time interval in which the differences between two treatments were statistically significant ($P < 0.05$) (Exp. 1)


70
60
50
40
30
20
10
0
mol/ 100 mol
0
1
2
3
4
Time after feeding (h)
Acetate
Propionate
FL
CTR
FL
CTR

a


70
60
50
40
30
20
10
0
mol/ 100 mol
49
56
63
70
Days of study
Acetate
Propionate
FL
CTR
FL
CTR

b

Figure 2: Changes in VFA concentration registered in response to a commercial flavonoid supplement (Bioflavex®) in 16 rumen-cannulated Fleckvieh heifers (Experiment 2) in relation to time since concentrate was administered [a, Interaction of treatment x time since concentrate was given) and over the course of the experiment (b, Interaction of treatment x days of experiment (sampling) for acetate and propionate (mol/100 mol)

LITERATURE CITED

AOAC. 1990. Official Methods of Analysis. 15th ed. Assoc. Off. Anal.Chem., Arlington, VA.

Bach, A., C. Iglesias, and M. Devant. 2007. Daily rumen pH pattern of loose-housed dairy cattle as affected by feeding pattern and live yeast supplementation. Anim. Feed Sci. Tech. 136: 146-153.

Balcells, J., J. A. Guada, J. M. Peiro, and D. S. Parker. 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. J. Chromatogr-Biomed. 575: 153-157.

Bauer, M. L., D. W. Herold, R. A. Britton, R. A. Stock, T. J. Klopfenstein, and D. A. Yates. 1995. Efficacy of laidlomycin propionate to reduce ruminal acidosis in cattle. J. Anim. Sci. 73: 3445-3454.

Beauchemin, K. A., and J. G. Buchanan-Smith. 1990. Effects of Fiber Source and Method of Feeding on Chewing Activities, Digestive Function, and Productivity of Dairy cows. J. Dairy Sci. 73: 749-762.

Bevans, D. W., K. A. Beauchemin, K. S. Schwartzkopf-Genswein, J. J. McKinnon, and T. A. McAllister. 2005. Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle. J. Anim. Sci. 83: 1116-1132.

Broudiscou, L. P., and B. Lassalas. 2000. Effects of Lavandula officinalis and Equisetum arvense dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40: 431-440.

Broudiscou, L. P., Y. Papon, and A. Broudiscou. 1999. Effects of inorganic nitrogen and amino acids on the degradation of ammonia-treated barley straw and proteosynthesis in a continuous culture of rumen microbes. Anim. Feed Sci. Tech. 77: 149-162.

Burrin, D. G., and R. A. Britton. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63: 888-893.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8: 130-132.

Clavel, T., G. Henderson, C. A. Alpert, C. Philippe, L. Rigottier-Gois, J. Doré, and M. Blaut. 2005. Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. Appl. Environ. Microb. 71: 6077-6085.

Coe, M. L., T. G. Nagaraja, Y. D. Sun, N. Wallace, E. G. Towne, K. E. Kemp, and J. P. Hutcheson. 1999. Effect of virginiamycin on ruminal fermentation in cattle during adaptation to a high concentrate diet and during an induced acidosis. J. Anim. Sci. 77: 2259-2268.

Cooper, R., and T. Klopfenstein. 1996. Effect of rumensin and feed intake variation on ruminal pH. page A1 in Scientific Update on Rumensin/tylan/micotil for the Professional Feedlot Consultant. Elanco Animal Health, Indianapolis, IN.

De Freitas, J. A., J. C. De Souza, R. P. Lana, R. P. Antonangelo, A. A. De Freitas, and R. T. S. De Santana. 2007. Ethanolic extract of propolis in lactating cows. J. Dairy Sci. 90(Suppl. 1): 105. (Abstr.)

Decroos, K., S. Vanhemmens, S. Cattoir, N. Boon, and W. Verstraete. 2005. Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. Arch. Microbiol. 183: 45-55.

Devant, M., A. Anglada, and A. Bach. 2007. Effects of plant extract supplementation on rumen fermentation and metabolism in young Holstein bulls consuming high levels of concentrate. Anim. Feed Sci. Tech. 137: 46-57.

Devant, M., A. Ferret, S. Calsamiglia, R. Casals, and J. Gasa. 2001. Effect of nitrogen source in high-concentrate, low-protein beef cattle diets on microbial fermentation studied in vivo and in vitro. J. Anim. Sci. 79: 1944-1953.

European Communities. 2003. Regulation EC No 1831/2003 of the European Parliament and Council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur. Union L268: 29-43.

France, J., and R. C. Siddons. 1993. Volatile Fatty Acid Production. page 196-203 in Quantilitive Aspects Ruminant Digestion and Metabolisim. J. M. Forbes and J. France ed. CAB International, Willingford, UK.

Gibson, G. R., E. R. Beatty, X. Wang, and J. H. Cummings. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108: 975-982.

Gladine, C., E. Rock, C. Morand, D. Bauchart, and D. Durand. 2007. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Brit. J. Nutr. 98: 691-701.

Goad, D. W., C. L. Goad, and T. G. Nagaraja. 1998. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. J. Anim. Sci. 76: 234-241.

González, L. A., L. B. Correa, A. Ferret, X. Manteca, J. L. Ruíz-de-la-Torre, and S. Calsamiglia. 2009. Intake, water consumption, ruminal fermentation, and stress response of beef heifers fed after different lengths of delays in the daily feed delivery time. J. Anim. Sci. 87: 2709-2718.

Harborne, J. B., and C. A. Williams. 2000. Advances in flavonoid research since 1992. Phytochemistry 55: 481-504.

Jouany, J. P. 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments. 2: 131-144.

Krehbiel, C. R., R. A. Britton, D. L. Harmon, T. J. Wester, and R. A. Stock. 1995. The effects of ruminal acidosis on volatile fatty acid absorption and plasma activities of pancreatic enzymes in lambs. J. Anim. Sci. 73: 3111-3121.

Martín-Orúe, S. M., J. Balcells, J. A. Guada, and M. Fondevila. 2000. Microbial nitrogen production in growing heifers: Direct measurement of duodenal flow of purine bases versus urinary excretion of purine derivatives as estimation procedures. Anim. Feed Sci. Tech. 88: 171-188.

Martín-Orúe, S. M., J. Balcells, F. Zakraoui, and C. Castrillo. 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: Effect of detachment procedure. Anim. Feed Sci.Tech. 71: 269-282.

Middleton Jr, E., C. Kandaswami, and T. C. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52: 673-751.

Nagaraja, T. G., T. B. Avery, S. J. Galitzer, and D. L. Harmon. 1985. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. Am. J. Vet. Res. 46: 2444-2452.

Ouwerkerk, D., A. V. Klieve, and R. J. Forster. 2002. Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92: 753-758.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in cattle: A review. J. Anim. Sci. 76: 275-286.

Pérez, J. F., J. Balcells, J. A. Guada, and C. Castrillo. 1997. Rumen microbial production estimated either from urinary purine derivative excretion or from direct measurements of 15N and purine bases as microbial markers: Effect of protein source and rumen bacteria isolates. Anim. Sci. 65: 225-236.

Rhodes, M. J. C. 1996. Physiologically-active compounds in plant foods: An overview. P. Nutr. Soc. 55: 371-384.

Russell, J. R., and T. Hino. 1985. Regulation of lactate production in Streptococcus bovis: A spiraling effect that contributes to rumen acidosis. J. Dairy Sci. 68: 1712-1721.

SAS. 2000. SAS User guide. Statistical analysis system institute Inc., Cary, NC.

Schelling, G. T. 1984. Monensin mode of action in the rumen. J.Anim. Sci. 58: 1518-1527.

Schoefer, L., R. Mohan, A. Braune, M. Birringer, and M. Blaut. 2002. Anaerobic C-ring cleavage of genistein and daidzein by Eubacterium ramulus. FEMS Microbiol. Lett. 208: 197-202.

Stack, R. J., R. E. Hungate, and W. P. Opsahl. 1983. Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8. Appl. Environ. Microb. 46: 539-544.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microb. 67: 2766-2774.

Taylor, K. A. C. C. 1996. A simple colorimetric assay for muramic acid and lactic acid. Appl. Biochem. Biotechnol. Enzym. Eng. Biotechnol. 56: 49-58.

Tzounis, X., J. Vulevic, G. G. C. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. Kwik-Uribe, and J. P. E. Spencer. 2008. Flavanol monomer-induced changes to the human faecal microflora. Brit. J. Nutr. 99: 782-792.

Van Niekerk, B. D., J. T. Reid, A. Bensadoun, and O. L. Paladines. 1963. Urinary creatine as an index of body composition. J. Nutr. 79: 463-473.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Winter, J., L. H. Moore, V. R. Dowell Jr, and V. D. Bokkenheuser. 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microb. 55: 1203-1208.

Wu, V. C. H., X. Qiu, B. G. de los Reyes, C. S. Lin, and Y. Pan. 2009. Application of cranberry concentrate (Vaccinium macrocarpon) to control Escherichia coli O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa. Food Microbiol. 26: 32-38.

Yaghoubi, S. M. J., G. R. Ghorbani, H. R. Rahmani, and A. Nikkhah. 2007. In vitro manipulation of rumen fermentation by propolis flavonoids and monensin. J. Dairy Sci. 90(Suppl. 1): 105-106. (Abstr.)

Yu, Z., and M. Morrison. 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microb. 70: 4800-4806.

CHAPTER 5

Effects of the flavonoid extract Bioflavex® or its pure components on rumen fermentation of intensively reared calves

The context of this chapter is submitted as a research article to the Journal of Animal Science (under peer-review, R2).

ABSTRACT

Two experiments were performed to study the effects of the citrus flavonoids extract Bioflavex (Interquim S.A., FerrerHealthTech, Sant Cugat, Barcelona, Spain; **BF**) on the fermentation characteristics of high concentrate diets. In an *in vivo* experiment, 8 Friesian calves (398 ± 12.2 kg body weight, BW) fitted with a rumen canula were given a basal concentrate (**CTR**) or CTR supplemented with BF (450 mg/kg dry matter, DM) in a 2×4 cross-over design. No differences were observed in concentrate and straw intake, average daily gain (ADG) or feed conversion ratio (FCR) between BF and CTR calves after 24 d of experiment. When rumen contents were sampled at 0, 4 and 8 h post feeding, it was observed that BF improved pH values (6.1 vs. 5.8, $P < 0.01$) and molar proportion of propionate (24.2 vs. 22.5 %, $P < 0.01$), whereas BF reduced lactate concentration (2.08 vs. 0.91 mmol/L, $P < 0.01$) and improved the relative abundance of lactate-consuming microorganism such as *Selenomomas ruminantium* ($P < 0.01$) and *Megaesphaera elsdenii* ($P = 0.06$), although did not alter that of the lactate-producing *Streptococcus bovis*. In an *in vitro* experiment, the effect of BF and its pure flavonoid components added to the mixture was studied, with inoculum from 4 steers given a high concentrate diet, in four experimental series. Bioflavex® and its main components such as naringine (**NG**), neohesperidine (**NH**) and poncirine (**PC**) were added to the incubation medium at 500 µg/g DM, with the unsupplemented substrate also included as a control (CTR). Up to 12 h post incubation, BF and its main components (NH and PC) reduced ($P < 0.01$) the volume of gas produced and the molar proportion of acetate ($P < 0.01$), increasing that of propionate ($P < 0.01$). PC reduced the relative quantification of *S. bovis*, whereas NH and BF increased the relative quantification of *M. elsdenii* in relation to CTR ($P < 0.01$). BF supplementation in calves receiving a high concentrate diet was effective in preventing collapses in pH and enhanced rumen fermentation efficiency through modifying the activity of lactating-consuming bacteria and modulating rumen fermentation towards a greater molar proportion of propionate and a reduction of that of acetate, which may suggest that flavonoid supplementation might have a role in modulating the activity of rumen microbiota.

Key words: flavonoids, Holstein calves, intensive beef production, *in vivo*, *in vitro*, rumen fermentation

INTRODUCTION

Manipulation of rumen fermentation to improve efficiency is the main aim of using feed additives in ruminant animals. Promoting the improvement of volatile fatty acids (VFA)

together with a reduction of ammonia rumen concentration and methane production are considered as desirable changes in rumen environment (Bodas et al., 2012). Antibiotic therapies have been used to alleviate rumen fermentation dysfunctions (i.e., acidosis or bloat) when high concentrate rations are given to improve rumen efficiency and animal performances (Beauchemin and Buchanan-Smith, 1990). However, massive environmental widespread of antibiotic associated with potential cross-over resistance to human therapies, together with consumers demands on food quality and safety promoted the ban of dietary antibiotic administration in Europe (European Communities, 2003). Removal of antibiotics conventionally used as feed additives forced nutritionists to explore some natural less aggressive substances as alternatives (Patra and Saxena, 2009). Secondary plant extracts (e.g. flavonoids) have been proposed as an alternative to antibiotic therapies (Rhodes, 1996). Balcells et al. (2012) previously showed that addition of Bioflavex® (BF; Interquim S.A., Barcelona, Spain), a blend of natural flavonoids extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*), was effective in alleviating reductions in rumen pH in heifers subjected to experimentally induced acidosis. It also improves rumen molar proportions of propionate and prevents lactate accumulation by creating conditions that favored lactate-consuming microorganisms such as *Megasphaera elsdenii*. The objectives of the present work were, i) to confirm *in vivo* the effects of Bioflavex® on rumen metabolism using rumen fistulated calves fed high concentrate rations and, ii) to estimate the contribution of each pure flavonoid as component of the commercial mixture in an *in vitro* system, to simulate *in vivo* rumen fermentation.

MATERIALS AND METHODS

In vivo trial

This experiment was carried out in the facilities of the Servicio de Experimentación Animal (SEA) 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 EU Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Eight crossed, 9 month old Holstein-Frisian calves (398 ± 12.2 kg) were fitted with a canula (88 mm length and 10 mm i.d. DIVASA Farmavic S.A, Vic, Barcelona, Spain) in the dorsal sac of the rumen. After recovery from canulation, animals were housed in 3.2 x 1.7 m individual pens with concrete floor, 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 with the commercial mixture of flavonoids Bioflavex® comprising mostly Naringine (NG, 200 g/kg), which is extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*) (400 g/kg composed mainly by Naringine **NG**; Neohesperidine; **NH**, , and Poncirine; **PC**, plus tracer amounts of Hesperidine; **HS** Isonaringina; **IN** and Neoeriocitrine; **NE**). The experiment lasted for 24 days and was organized in a 2×4 cross over design, with two periods of 12 days and four calves per period and treatment. Concentrate and straw were supplied *ad libitum* in separated feeders, the former in only one dose (at 0800 h) and the latter in three daily doses, and consumptions and orts were daily recorded on DM basis. Calves were weighed individually when starting and at the end of each period. The last two days of each experimental period, 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 (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 ° C until further molecular analysis. Other two 4 mL subsamples were pipetted to tubes containing either 1 mL of a solution made up with 20 mL/L ortophosphoric acid and 2 mL/L of 4-methylvaleric acid as internal marker or 4 mL of HCl 0.2 *N*, that were stored at -20 ° C until further analysis of volatile fatty acids (VFA) and ammonia (NH_3) concentration, respectively.

In vitro trial

Four incubations series were conducted to evaluate the effect of the Bioflavex® mixture (BF) or its pure flavonoid components (NG; NH, PC, HS, IN and NE) included at 500 µg/g DM of substrate, on *in vitro* rumen fermentation (Theodorou et al., 1994) using rumen liquor from four rumen cannulated growing steers fed a high concentrate diet (90 % of a commercial concentrate and 10 % barley straw; Table 1) given *ad libitum* for at least 4 weeks. For each incubation series, the rumen contents were collected from a different animal, filtered through a double layer of gauze and used immediately as inoculum (10 % of total incubation volume). Serum glass bottles of 120 mL total volume (four bottles per 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. A mixture of 600 mg of the same concentrate given to steers plus 60 mg barley straw was used as substrate. 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 ° C in a shaking water bath for 12 h.

Pressure measurements were determined with a TP704 Manometer (DELTA OHM, Italy) at 2, 4, 6, 8, 10 and 12 h after the onset of the 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 DM. At the onset (Time 0) of each incubation series, two samples were taken from the 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 were weighed, immediately frozen in liquid N and stored at -80 ° C for microbiota analyses. The remaining content was filtered through a metal sieve (1 mm mesh size) and sampled for subsequent analyses of ammonia nitrogen and VFA concentration, as above. Samples were immediately frozen (-20 º C) until further analyses.

Microbiological and chemical analyses

The DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions. Relative abundance of *Streptococcus bovis*, *Selenomonas ruminantium* and *Megasphaera elsdenii* in relation to the total bacteria were determined using specific primers. The relative quantification was carried out using $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Analyses were performed on CFX96 Touch real-time PCR detection system (BioRad, Laboratories Inc., Hercules, CA, USA). The primer sets and qPCR condition were described in Table 2.

The DM content of the concentrate and straw was determined at 105 ° C until a constant sample weight. Ash content was determined by incineration on muffle furnace (550 ° C for 4 h) and crude protein (CP) was analyzed according to AOAC (1990). The proportion of neutral detergent fiber (NDF) was determined according Van Soest et al. (1991) procedures, using alpha amylase, and discounting ashes from the residue. Ammonia concentration was determined by the Chaney and Marbach (1962) method after sample centrifugation (2500 x g, 20 min). The VFA concentrations were determined by gas chromatography (GC), based on the technique proposed by Jouany (1982) using a capillary column (BP21 30 m x 0.25 mm ID x 0.25 µm, DE, USA). Lactate was measured using the method of Taylor (1996).

Statistical Analysis

In vivo rumen fermentation parameters (pH, ammonia and VFA concentration and proportions of bacterial species) were analyzed as a 2×4 cross over design using the mixed

procedure of SAS (SAS, Inst. Inc., Cary, NC). The model included phase, treatment, sampling day and time post-feeding (hours, considered as repeated measures) and their interactions as fixed factors. Animal within period was considered as the experimental unit.

Results from the *in vitro* trial were studied as a completely randomized block design, occurring in 4 different blocks. Each animal donor, which agrees with the incubation series, was considered as the experimental unit. Computations were performed using the mixed procedure of SAS (SAS, Inst. Inc., Cary, NC), each experiment series being considered as a random effect. The model included block, treatment and their interactions as fixed factors.

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

RESULTS

In vivo trial

There were no differences between CTR and BF in daily intake of concentrate (7.42 vs. 7.79 kg DM/d, SEM = 0.28) or straw (0.83 vs. 1.05 kg DM/d, SEM = 0.16), nor in weight gain (1.4 vs. 1.4 kg/d, SEM = 0.12) along the experimental period. Mean values of pH, ruminal NH_3 and VFA concentrations and relative molar VFA proportions are presented in Table 3 The average pH was higher ($P < 0.01$) in calves given BF than CTR (6.1 vs. 5.8; SEM = 0.05), and it dropped significantly 4 h after feeding ($P < 0.01$). No interaction between both factors was recorded. Average concentration of NH_3 was higher in CTR ($P < 0.01$), and it was reduced after feeding ($P < 0.01$). Total VFA concentration was not affected by the flavonoid supplementation, but it increased from 0 to 4 h after feeding ($P < 0.01$). Despite molar proportion of propionate increased with flavonoid addition ($P = 0.05$), this only occur at 0 h, whereas no treatment differences were recorded at 4 and 8 h (interaction treatment x hour; $P < 0.01$; Fig. 1a). The opposite trend occurred with acetate proportion, that was lower at 0 h with BF but did not differ afterwards (interaction treatment x hour; $P < 0.01$; Fig. 1b). No treatment differences were recorded for molar proportions of the other VFAs, despite that of butyrate increased, and isobutyrate and isovalerate decreased, from 0 to 4 h ($P < 0.01$).

Rumen lactate concentration and relative proportions of the lactate-producing bacteria *S. bovis* and the lactate-consuming bacteria *S. ruminantium* and *M. elsdenii*, are presented in Table 4. 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, but it decreased linearly with time (P = 0.016). In contrast, the average abundance of *M. elsdenii (*P = 0.06) and *S. ruminantium* (P < 0.01) increased with BF. However, whereas concentration of *M. elsdenii* increased with time (P = 0.02), that of *S. ruminantium* showed an unequal evolution: no differences among treatments were observed at 0 and 2 hours after feeding, but at 8 hours relative proportion *S. ruminantium* increase in BF supplemented animals whereas decrease in CTR (Interaction treatment x hour; P < 0.05).

In vitro trial

In vitro gas production values from the six selected times of incubation are presented in Table 5. Addition of BF to the culture media reduced gas production (P < 0.01) in relation to the CTR, as it was also the case for its main components NH and PC (P < 0.05). Although NG also reduced numerically gas production, its effect did not reach statistical significance. Tracer components did not alter gas production in relation to values recorded with CTR bottles.

Table 6 shows ammonia and VFA concentrations of the 12 h *in vitro* incubation trial. Addition of flavonoids has a small effect on NH_3 concentration, and only a slight but significant (P = 0.03) reduction in bottles supplemented with the tracer component IN was detected. No treatment differences were recorded (P > 0.10) on the total VFA concentration, nor on molar proportions of butyrate and the minor VFAs. However, flavonoid supplementation increased (P < 0.01) the molar proportion of propionate at the expense of acetate for either BF or each one of its components, in relation to CTR.

The numerical differences observed in lactate concentrations after 12 h incubation *in vitro* in BF and its main components NG and NH compared with CTR did not reach statistical significance (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 leads to a rumen pH reduction and may induce sub-acute (pH 5.6 to 6.0, Goad et al., 1998) or acute (pH below 5.6, Nagaraja et al., 1985) acidosis. Rumen acidity is provoked by a reduction in salivation, due to shortage of fiber size, and an increase in VFA production, including lactic acid accumulation. Moreover,

starch availability improves the presence of glucose-utilizing bacteria through the lactate pathway (such as *S. bovis*) and the higher lactate concentration may consequently increase to a certain extent the titers 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, BF addition was effective in preventing pH reduction (Balcells et al., 2012); however, in a simultaneous trial using growing heifers the positive effect of the citrus flavonoids blend was less conclusive at the rumen metabolism level and no improvement in ADG was detected. This more controlled in vivo assay using cannulated animals may improve the understanding of BF effect on rumen environment, and the *in vitro* simulation trial of rumen fermentation allows for identify the effectiveness of the pure flavonoid components into the BF mixture.

Calves consumed 88 to 90% concentrate in diet, and consequently rumen pH values dropped to sub-acute values from 4 to 8 h after feeding (Goad et al., 1998). Allowing the animals to adapt to the high-grain diets and the low degradability of corn starch might probably reduce the possibility of acute acidosis (Owens et al., 1998). Average recorded pH and its daily evolution in CTR calves agrees well with previous reports under similar experimental conditions (Beauchemin et al., 2001; Koenig et al., 2003). Confirming our previous work (Balcells et al., 2012), BF addition increased average pH on 0.3 pH units, and BF fed calves apparently tended to a faster recovery of normal pH values after concentrate feeding than those given CTR (5.70 vs. 5.34 at 8 h post-feeding, SEM = 0.08; $P < 0.05$).

It is logical to expect that the effect of BF on microbial environment should relay on the activity of the main flavonoid components of the mixture (i.e., NG, NH and PC). Effectively, NG and NH reduced gas production and microbial activity at a similar level. Moreover, the relationship between 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. This fact would confirm previous results from our group (Seradj et al., 2014b), who cannot demonstrate any positive effect between over-dosage of flavonoids and methane production under similar experimental conditions.

Anti-microbial activity of flavonoids on pathogenic (Wu et al., 2009) and nonpathogenic (Broudiscou and Lassalas, 2000) bacteria have been reported, and a hypothetical mechanism through the cleavage of C-ring in the flavonoids to produce the toxic phenolic acids has been described (Schoefer et al., 2002). The degradation of NG (as the main component of BF) to the corresponding aglycone (Gladine et al., 2007) and further C-ring cleavage to produce

anti-microbial compounds (Winter et al., 1989) would explain some anti-microbial effect related to both NG and BF. Although it is true that the improvement in pH values and the significant reduction in gas production may promote a reduction in rumen microbial fermentation, such anti-microbial activity must have some specificity, given that titters of lactate-consuming microorganism were not reduced, but even increased. Authors are not aware of any anti-microbial metabolic pathway for NH but probably the similarity between both flavonoid structures may suggest a similar metabolic pathway.

Rumen NH_3 concentration reflects the balance between production (mostly from protein degradation) and the summation of microbial utilization 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 improving duodenal N flow, which suggest an improvement in microbial N usage (Balcells et al., 2012). However, the fact that differences in NH_3 concentration have a minor relevance *in vitro* suggests that the recorded differences *in vivo* may be explained though differences in absorption mechanism.

Bioflavex® was able to partially buffer rumen pH. This seems to be clearly related with the drop in lactate concentration with BF, although this effect was not consistently supported by the recorded values in rumen VFA concentration *in vivo* Rumen pH values are primarily determined by fermentation end-products (i.e. VFA, lactate, NH_3; (Owens et al., 1998), where VFA concentration only explains 32 % of the total observed variation in rumen pH (Sauvant et al., 1999) and changes in feed intake, saliva production, dietary buffers, and rate of passage may alter such relationship. In the *in vitro* trial, the bottles supplemented with flavonoids showed a lower numerical VFA concentration but differences did not reach significance.

In both trials, BF supplementation consistently enhanced rumen molar proportion of propionate at the expense of acetate, and thus reduced the acetate-to-propionate ratio. In this sense, it is necessary to remark that rumen concentration and molar proportion of VFAs are indexes of the equilibrium between production and absorption of the end product. However, activity of BF and its components on molar proportions of propionate and acetate was confirmed in the *in vitro* trial, where the absorption process is excluded. Tissues use propionate more efficiently than acetate, and besides propionate may enter in the citric acid cycle in order to produce oxaloacetate, which can then be used to produce glucose via gluconeogenesis. However, it has been demonstrated that differences in rumen fermentation

may have a small effect on performances of animals fed high concentrate diets (Schelling, 1984).

In both *in* vivo and *in* vitro trials, supplementation with BF and the flavonoid compounds changed the VFA fermentation profile, which suggests that flavonoids might have modified the rumen microbiota. This has been confirmed in the human gut, where genistein is able to modify prevalent species of microbiota (Clavel et al., 2005; Schoefer et al., 2002). The specific effect of flavonoids has also been demonstrated in the rumen ecosystem, such as that of catequin 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 ciliate protozoa and archaeas such as hydrogenotrophic methanogens and *Methanosarcina ssp.* (Seradj et al., 2014a). In our study, the inclusion of BF in diet did not alter the rumen abundance of lactate-producing bacteria such as *S. bovis*, but tended improved that of the lactate-consuming species *M. elsdenii* ($P = 0.06$) and *S. ruminantium* ($P < 0.01$), although no changes in abundance of *S. ruminantium* were previously detected (Balcells et al., 2012). Differences in the experimental protocol and the acidity level in the media may probably explain this discrepancy.

The *in vitro* trial support results from *in vivo:* addition of BF improved 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 homogenous, and BF and its main components improved its concentration *in vitro*.

A negative relationship between rumen pH values and lactate concentrations was observed in the *in vivo* trial, pH decreasing after concentrate feeding according to lactate accumulation (Owens et al., 1998). However, only numerical differences in lactate concentration were detected in CTR bottles when the effect of BF and its components were tested *in vitro*, suggesting that lactate absorption under sub-acute acidosis conditions may plays a crucial role in lactate accumulation *in vivo*. Nevertheless, a different behavior in lactate metabolism between the *in vivo* and *in vitro* could not be discarded. Lactate accumulation during acidosis induction may vary largely, exceeding to 50 mmol/L in acute acidosis (Nagaraja et al., 1985) although values lower than 10 mmol/L have been described during subacute acidosis conditions (Balcells et al., 2012; Burrin and Britton, 1986; Goad et al., 1998) or even cases in which concentration was unaffected (Bevans et al., 2005). In our experiment, when sub-acute pH conditions were reached after feeding, a significant lactate accumulation was detected.

Moreover, during acidosis challenge S. *bovis* titters increased, its 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 recorded with BF was confirmed *in vitro*, where a positive effect of BF and its main components on the lactate-consuming bacteria titters was evidenced.

In summary, BF supplementation in high concentrate fed calves was effective in preventing pH reduction and enhancing rumen fermentation efficiency through modifying the activity of lactating-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 might have a role in activity of rumen microbiota.

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

Item (%)	Diets	
	CTR	BF
Barley straw		
Concentrate		
Corn grain	35	35
Barley grain	25	25
Soybean Meal (44 %)	10	10
Wheat Bran	6	6
Sunflower meal (30 %)	3.5	3.5
Gluten Feed (20 %)	8	8
Beet Pulp	7	7
Palm oil	2.5	2.5
Calcium	1.3	1.3
bi-calcium phosphate	0.8	0.8
Salt	0.3	0.3
Mineral and vitamin premix	0.4	0.4
Sepiolite	0.2	0.2
Bioflavex® (mg/kg)	0	450
Chemical Composition (g/kg DM)		
	Concentrate	Barley Straw
DM	90.61	89.04
OM	93.16	93.35
CP	15.43	3.78
NDF	29.26	74.41

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 used in the Experiment.

Target	Author	Primers	
		Forward	Reverse
Quantitative PCR			
†Total bacteria	(Maeda et al., 2003)	5´-GTGSTGCAYGGYTGTCGTCA-3´	5´-ACGTCRTCCMCACCTTCCCC-3´
†Selenomonas ruminantium	(Tajima et al., 2001)	5´-TGCTAATACCGAATGTTG-3´	5´-TCCTGCACTCAAGAAAGA-3´
†Streptococcus bovis	(Tajima et al., 2001)	5´-CTAATACCGCATAACAGCAT-3´	5´-AGAAACTTCCTATCTCTAGG-3´
†Megasphaera elsdenii	(Ouwerkerk et al., 2002)	5´-GACCGAAACTGCGATGCTAGA-3´	5´-CGCCTCAGCGTCAGTTGTC-3´

†qPCR conditions: 1 x (95 º C 10:00 min), 40 x (95 º C 00:15 min, 60 º C 00:10 min, 72 º C 00:55 min) + Melt Curve

Efficiency for all the used primes were between 90 to 95 %

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

Items	Treatments		SEM	Hours			SEM	*P* value[1]		
	CTR	BF		0	4	8		Tr	H	Tr x H
pH	5.8	6.1	0.05	6.9	5.4	5.5	0.06	<.01	<.01	0.37
NH3, mg /L	28.0	14.7	2.80	40.0	8.4	15.7	3.30	<.01	<.01	0.17
VFA, mM	92.1	96.7	3.84	60.6	113.8	108.7	4.53	0.41	<.01	0.37
VFA, mol/100 mol										
Acetate	61.9	60.6	0.68	64.5	60.1	59.3	0.80	0.19	<.01	0.01
Propionate	22.5	24.2	0.60	21.0	24.4	24.7	0.71	0.05	<.01	<.01
Butyrate	10.7	10.2	0.44	9.0	10.9	11.4	0.52	0.44	<.01	0.92
Iso-Butyrate	1.0	1.0	0.05	1.29	0.89	0.81	0.05	0.89	<.01	0.90
Valerate	2.7	2.6	0.12	2.46	2.65	2.81	0.14	0.36	0.20	0.41
Iso-Valerate	1.1	1.3	0.11	1.71	1.01	0.97	0.132	0.15	<.01	0.75

Bioflavex® (BF; Interquim S. A. (FerrerHealthTech), Sant Cugat, Barcelona, Spain) and Control (CTR)

[1]Tr = treatment effect; H = time post feeding; Tr x H = interaction of the treatment and time post feeding

Table 4 Effects of Bioflavex® on rumen lactate concentrations (mmol/L), and relative quantification of *Streptococcus bovis*, *Selenomonas ruminantium*, *Megasphaera elsdenii* and over the post feeding hours.

Items	Treatments		SEM	Hours			SEM	*P* value[1]		
	CTR	BF		0	4	8		Tr	H	Tr x H
Lactate, mmol/L	2.08	0.91	0.109	0.81	1.71	1.83	0.38	<.01	<0.01	0.22
Relative Quantification										
S. bovis	0.10	0.09	0.007	0.12	0.09	0.08	0.009	0.80	0.016	0.96
M. elsdenii	0.02	0.05	0.011	0.01	0.04	0.06	0.013	0.06	0.02	0.19
S. ruminantium	2.4	3.3	0.19	2.9	2.8	2.9	0.22	<.01	0.99	0.06

†$2^{(-\Delta Ct)} \times 10^2$

[1]Tr = treatment effect; H = time post feeding; Tr x H = interaction of the treatment and time post feeding

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, un-supplemented (CTR) or supplemented with Bioflavex® (BF) or its pure flavonoids components

Time (hour)	**Treatments**									
	CTR	BF	*BF-Major Components*			*BF-Tracer Components*			SEM	*P* value
			NG	NH	PC	HS	IN	NE		
2	47.7^{ab}	39.9 ^{bc}	25.4 ^{d}	37.3 ^{bc}	40.2 ^{bc}	51.8 ^{a}	43.3 ^{abc}	35.9 ^{c}	2.09	
4	92.5 ^{ab}	85.8 ^{bc}	83.2 ^{bcd}	75.7 ^{d}	81.5 ^{cd}	96.9 ^{a}	85.8 ^{bc}	89.7 ^{abc}	1.91	
6	126.8 ^{abc}	119.8 ^{cd}	120.0 ^{bcd}	104.8 ^{e}	113.3 ^{de}	130.4 ^{a}	120.2 ^{bcd}	128.9 ^{ab}	1.87	<.01
8	153.2 ^{ab}	143.5 ^{cd}	149.6 ^{bcd}	131.0 ^{e}	142.0 ^{d}	151.4 ^{abc}	147.3 ^{bcd}	159.5 ^{a}	1.90	
12	173.8 ^{ab}	162.7 ^{c}	166.9 ^{bc}	149.3 ^{d}	161.2 ^{c}	168.8 ^{abc}	165.1 ^{bc}	179.1 ^{a}	2.06	

Different superscripts $^{(a,b,c)}$ denote statistical differences ($P < 0.05$) among treatments

Bioflavex® (BF), Naringine (NG), Neohesperidine (NH), Poncirine (PC), Hesperidine (HS), Isonaringine (IN) , Neoeriocitrine (NE), , and Control (CTR)

Table 6. *In vitro* fermentation parameters in response to flavonoid mixture (Bioflavex®) and its pure flavonoid components

Items	Treatments								SEM	*P* value
			BF-Major Components			*BF-Tracer Components*				
	CTR	BF	NG	NH	PC	HS	IN	NE		
Parameters										
NH3-N (mg/L)	158.8 [a]	143.8 [ab]	149.0 [ab]	146.1 [ab]	140.5 [ab]	152.5 [ab]	135.9 [b]	150.1 [ab]	10.21	0.04
VFA, mM	37.5	34.7	31.9	33.4	33.4	33.4	33	34.6	2.97	0.43
VFA, mol/100 mol										
Acetate	58.9 [a]	53.6 [b]	51.3 [b]	52.0 [b]	52.5 [b]	53.2 [b]	51.5 [b]	52.2 [b]	1.68	<.01
Propionate	25.5 [b]	30.3 [a]	33.0 [a]	31.9 [a]	32.4 [a]	30.7 [a]	33.7 [a]	31.8 [a]	1.94	<.01
Butyrate	10.2	10.3	9.8	10.4	9.7	10.6	9.4	10.1	0.69	0.9
Iso-Butyrate	1.9	2	2	2	1.8	1.9	1.7	2	0.21	0.3
Valerate	2	2.1	2.1	2.1	2.1	2.1	2.2	2.3	0.13	0.33
Iso-Valerate	1.5	1.7	1.7	1.7	1.6	1.7	1.6	1.7	0.35	0.9

Different superscripts [(a,b,c)] denote statistical differences ($P < 0.05$) among treatments

Bioflavex® (BF), Naringine (NG), Neohesperidine (NH), Poncirine (PC), Hesperidine (HS), Isonaringine (IN) , Neoeriocitrine (NE), , and Control (CTR)

Table 7. Effects of Bioflavex® on lactate concentration (mmol/L) and relative quantification of *Streptococcus bovis*, *Selenomonas ruminantium*, *Megasphaera elsdenii* over the incubation time.

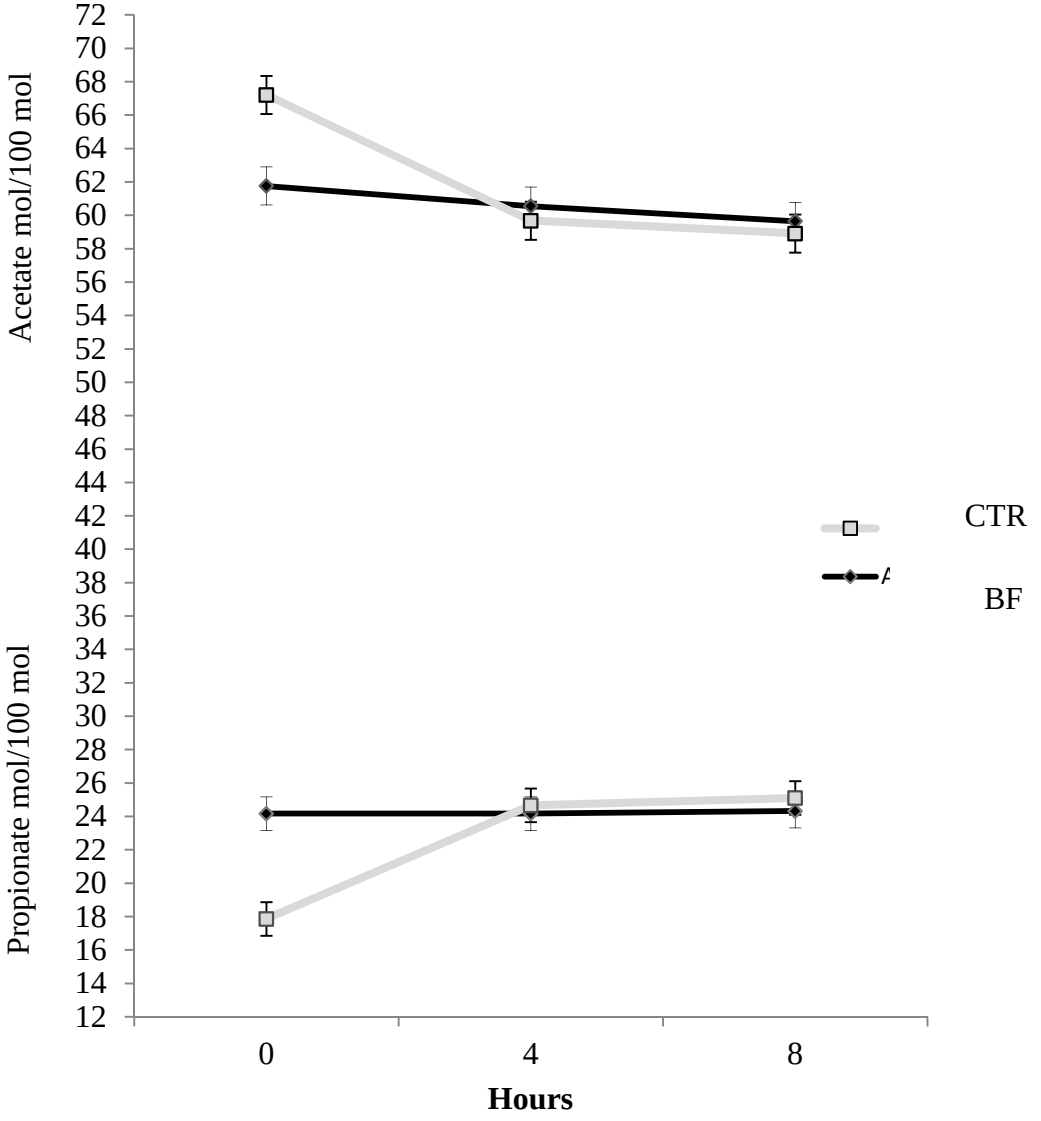
Items	Treatments									
	CTR	BF	*BF-Major Components*			*BF-Tracer Components*			SEM	*P* value
			NG	NH	PC	HS	IN	NE		
Lactate, mmol/L	0.81	0.45	0.55	0.5	0.98	0.48	1.02	1.35	0.07	0.1
Relative Quantification										
S. bovis†	0.015 ^{b}	0.014 ^{b}	0.013 ^{bc}	0.002 ^{d}	0.005 ^{cd}	0.014 ^{b}	0.005 ^{cd}	0.032 ^{a}	0.0015	<.01
M. elsdenii‡	0.8 ^{b}	4.9 ^{a}	4.4 ^{ab}	5.7 ^{a}	3.1 ^{ab}	1.1 ^{b}	3.6 ^{ab}	1.3 ^{b}	0.67	<.01
S. ruminantium†	0.1 ^{b}	0.5 ^{ab}	0.4 ^{ab}	0.2 ^{ab}	0.4 ^{ab}	0.6 ^{a}	0.5 ^{ab}	0.1 ^{b}	0.08	0.01

†$2^{(-\Delta Ct)} \times 10^{2}$

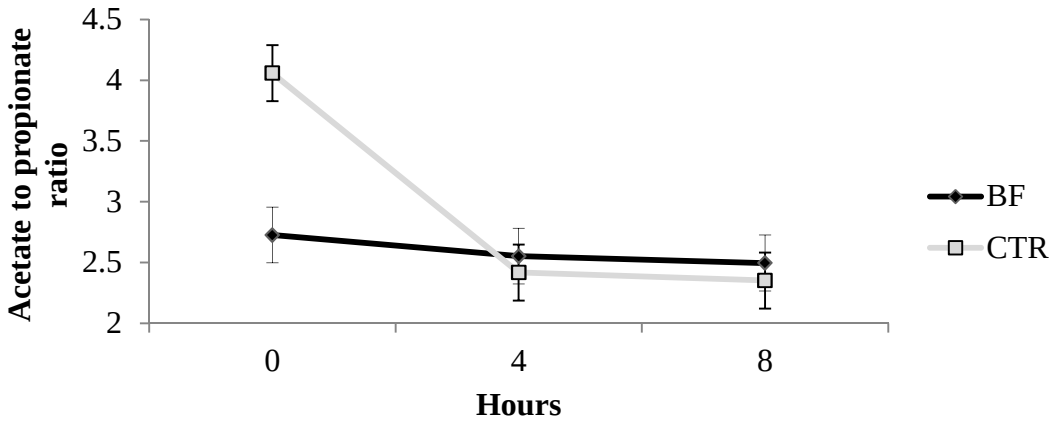
‡$2^{(-\Delta Ct)} \times 10^{6}$

Different superscripts $^{(a,b,c)}$ denote statistical differences ($P < 0.05$) among treatments

Bioflavex® (BF), Naringine (NG), Neohesperidine (NH), Poncirine (PC), Hesperidine (HS), Isonaringine (IN) , Neoeriocitrine (NE), , and Control (CTR)


Acetate mol/100 mol
Propionate mol/100 mol
72
70
68
66
64
62
60
58
56
54
52
50
48
46
44
42
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
0
4
8
Hours
CTR
BF

a


Acetate to propionate ratio
4.5
4
3.5
3
2.5
2
0
4
8
Hours
BF
CTR

b

Figure 1. Changes in molar proportion of acetate and propionate(a) registered in response to a commercial flavonoid supplement (Bioflavex®; Interquim S.A. (FerrerHealthTech), Sant Cugat, Barcelona, Spain) in 8 rumen-cannulated (b) together with the acetate to propionate ratio in relation to time post feeding (interaction of treatment × time post feeding).

LITERATURE CITED

AOAC. 1990. Official Methods of Analysis. 15th ed. Assoc. Off. Anal.Chem., Arlington, VA.

Balcells, J., A. Aris, A. Serrano, A. R. Seradj, J. Crespo, and M. Devant. 2012. Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. J. Anim. Sci. 90: 4975-4984.

Beauchemin, K. A., and J. G. Buchanan-Smith. 1990. Effects of Fiber Source and Method of Feeding on Chewing Activities, Digestive Function, and Productivity of Dairy Cows. J. Dairy Sci. 73: 749-762.

Beauchemin, K. A., W. Z. Yang, and L. M. Rode. 2001. Effects of barley grain processing on the site and extent of digestion of beef feedlot finishing diets. J. Anim. Sci. 79: 1925-1936.

Bevans, D. W., K. A. Beauchemin, K. S. Schwartzkopf-Genswein, J. J. McKinnon, and T. A. McAllister. 2005. Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle. J. Anim. Sci. 83: 1116-1132.

Bodas, R., N. Prieto, R. García-González, S. Andrés, F. J. Giráldez, and S. López. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Tech. 176: 78-93.

Broudiscou, L. P., and B. Lassalas. 2000. Effects of Lavandula officinalis and Equisetum arvense dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40: 431-440.

Broudiscou, L. P., Y. Papon, and A. F. Broudiscou. 2002. Effects of dry plant extracts on feed degradation and the production of rumen microbial biomass in a dual outflow fermenter. Anim. Feed Sci. Tech. 101: 183-189.

Burrin, D. G., and R. A. Britton. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63: 888-893.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8: 130-132.

Clavel, T., G. Henderson, C. A. Alpert, C. Philippe, L. Rigottier-Gois, J. Doré, and M. Blaut. 2005. Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. Appl. Environ. Microb. 71: 6077-6085.

European Communities. 2003. Regulation EC No 1831/2003 of the European Parliament and Council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur. Union L268: 29-43.

Gibson, G. R., E. R. Beatty, X. Wang, and J. H. Cummings. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108: 975-982.

Gladine, C., E. Rock, C. Morand, D. Bauchart, and D. Durand. 2007. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Brit. J. Nutr. 98: 691-701.

Goad, D. W., C. L. Goad, and T. G. Nagaraja. 1998. Ruminal Microbial and Fermentative Changes Associated with Experimentally Induced Subacute Acidosis in Steers. J. Anim. Sci. 76: 234-241.

Jouany, J. P. 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments. 2: 131-144.

Koenig, K. M., K. A. Beauchemin, and L. M. Rode. 2003. Effect of grain processing and silage on microbial protein synthesis and nutrient digestibility in beef cattle fed barley-based diets. J. Anim. Sci. 81: 1057-1067.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 -ΔΔCT method. Methods 25: 402-408.

Maeda, H., C. Fujimoto, Y. Haruki, T. Maeda, S. Kokeguchi, M. Petelin, H. Arai, I. Tanimoto, F. Nishimura, and S. Takashiba. 2003. Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. FEMS Immunol. Med. Mic. 39: 81-86.

Mould, F. L., R. Morgan, K. E. Kliem, and E. Krystallidou. 2005. A review and simplification of the in vitro incubation medium. Anim. Feed Sci. Tech. 123-124 Part 1: 155-172.

Nagaraja, T. G., T. B. Avery, S. J. Galitzer, and D. L. Harmon. 1985. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. Am. J. Vet. Res. 46: 2444-2452.

Ouwerkerk, D., A. V. Klieve, and R. J. Forster. 2002. Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92: 753-758.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in Cattle: A Review. J. Anim. Sci. 76: 275-286.

Patra, A. K., and J. Saxena. 2009. Dietary phytochemicals as rumen modifiers: A review of the effects on microbial populations. Anton. Leeuw. Int. J. G. 96: 363-375.

Rhodes, M. J. C. 1996. Physiologically-active compounds in plant foods: An overview. P. Nutr. Soc. 55: 371-384.

Sauvant, D., F. Meschy, and D. Mertens. 1999. Les composantes de l'acidose ruminale et les effets acidogènes des rations. INRA Prod. Anim. 12: 49-60.

Schelling, G. T. 1984. Monensin mode of action in the rumen. J. Anim. Sci. 58: 1518-1527.

Schoefer, L., R. Mohan, A. Braune, M. Birringer, and M. Blaut. 2002. Anaerobic C-ring cleavage of genistein and daidzein by Eubacterium ramulus. FEMS Microbiol. Lett. 208: 197-202.

Seradj, A. R., J. Crespo, M. Fondevila, and J. Balcells. 2014a. The effect of a citrus extract rich in flavonoids (Bioflavex) and its main components on rumen fermentation and microbial population under in vitro system using steers fed high concentrate diet as rumen liquor donors Joint Annual Meeting, Kansas City, US.

Seradj, A. R., J. Crespo, D. Villalba, and J. Balcells. 2014b. Inhibition of rumen methanogenesis induced by Bioflavex and its pure flavonoid components under in vitro fermentation using rumen fluid from steers fed high concentrate diets Joint Annual Meeting. ADSA-ASAS, Kansas City, US.

Stack, R. J., R. E. Hungate, and W. P. Opsahl. 1983. Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8. Appl. Environ. Microb. 46: 539-544.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-Dependent Shifts in the Bacterial Population of the Rumen Revealed with Real-Time PCR. Appl. Environ. Microb. 67: 2766-2774.

Taylor, K. A. C. C. 1996. A simple colorimetric assay for muramic acid and lactic acid. Appl. Biochem. Biotechnol. Enzym. Eng. Biotechnol. 56: 49-58.

Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48: 185-197.

Tzounis, X., J. Vulevic, G. G. C. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. Kwik-Uribe, and J. P. E. Spencer. 2008. Flavanol monomer-induced changes to the human faecal microflora. Brit. J. Nutr. 99: 782-792.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Winter, J., L. H. Moore, V. R. Dowell Jr, and V. D. Bokkenheuser. 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microb. 55: 1203-1208.

Wu, V. C. H., X. Qiu, B. G. de los Reyes, C. S. Lin, and Y. Pan. 2009. Application of cranberry concentrate (Vaccinium macrocarpon) to control Escherichia coli O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa. Food Microbiol. 26: 32-38.

CHAPTER 6

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.

The context of this chapter is published as a research article in Animal Feed Science and Technology (Volume 197, Pages 85-91) with DOI number: 10.1016/j.anifeedsci.2014.08.013.

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 µg/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 propionate 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.

Key words: flavonoids; *in vitro* incubation; gas production; methanogenesis.

1. 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 should be determined by the synergistic and/ or antagonistic action of the component mixture (*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.

2. Materials and methods

2.1. Incubation procedure

The effect of Bioflavex® (BF) and its pure flavonoid components (Hesperidine, HS; Isonaringine, IN; Naringine, NG; Neoeriocitrine, NE; Neohesperidine, NH and Poncirine, PC) on

rumen fermentation were tested against the control (CTR) at 200 μg/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 inoculum 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″/2 L, Hamilton syringe Gastight®, Nevada, USA) and immediately analyzed for methane concentration.

2.2. 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 μm, 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.

2.3. 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 and 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 $2^{-\Delta Ct}$ (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.

2.4. 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.

3. 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 consuming 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).

4. 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 and (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 and 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 depression 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.

5. 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.

Acknowledgements

This research was financed by Interquim S. A. (Ferrer Health Tech), Sant Cugat, Barcelona, Spain.

The authors would like to thank the staff of the Animal Nutrition lab and Dr. M. Llovera and Dr. T. Capell for their co-operation.

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

†1 x (95ºC 10:00min), 40 x (95ºC 00:15min, 60ºC 00:10min, 72ºC 00:55min)

¥1 x (95ºC 10:00min), 40 x (95ºC 00:15min, 57ºC 00:10min, 72ºC 00:55min)

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

	Treatments									
Items	BF	HS	IN	NG	NE	NH	PC	CTR	SEM	*P* value
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 $^{(a,b,c)}$ 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 (NH3-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
NH3-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 $^{(a,b,c)}$ 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

$^{\dagger}2^{(-\Delta Ct)} \times 10^{2}$; $^{\ddagger}\ 2^{(-\Delta Ct)} \times 10^{6}$

Different superscripts $^{(a,b,c)}$ 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)

References

AOAC, 2005. Official Methods of Analysis, 18th ed., W. Horwitz and G.W. Latimer (eds.), Association of Official Analytical Chemists, Gaithersburg, MA, USA.

Balcells, J., Aris, A., Serrano, A., Seradj, A.R., Crespo, J., Devant, M., 2012. Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. J. Anim. Sci. 90, 4975-4984.

Beuvink, J., Spoelstra, S., 1992. Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro. Appl. Microbiol. Biotechnol. 37, 505-509.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F.J., López, S., 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Tech. 176, 78-93.

Broudiscou, L.-P., Papon, Y., Broudiscou, A.F., 2000. Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes. Anim. Feed Sci. Tech. 87, 263-277.

Broudiscou, L.P., Lassalas, B., 2000. Effects of Lavandula officinalis and Equisetum arvense dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40, 431-440.

Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8, 130-132.

Chen, M., Wolin, M.J., 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microb. 38, 72-77.

Cushnie, T.P.T., Lamb, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Ag. 38, 99-107.

De Freitas, J.A., De Souza, J.C., Lana, R.P., Antonangelo, R.P., De Freitas, A.A., De Santana, R.T.S., 2007. Ethanolic extract of propolis in lactating cows. J. Dairy sci. 90(suppl. 1), 105.

Demeyer, D.I., Van Nevel, C.J., 1975. Methanogenesis, an integrated part of carbohydrate fermentation, and its control, Digestion and Metabolism in the Ruminant, pp. 366-382.

Denman, S.E., Tomkins, N.W., McSweeney, C.S., 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol. Ecol. 62, 313-322.

Franke-Whittle, I.H., Goberna, M., Insam, H., 2009. Design and testing of real-time PCR primers for the quantification of Methanoculleus, Methanosarcina, Methanothermobacter, and a group of uncultured methanogens. Can. J. Microbiol. 55, 611-616.

Garcia-Lopez, P.M., Kung, L., Odom, J.M., 1996. In vitro inhibition of microbial methane production by 9,10-anthraquinone. J. Anim. Sci. 74, 2276-2284.

Gladine, C., Rock, E., Morand, C., Bauchart, D., Durand, D., 2007. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Brit. J. Nutr. 98, 691-701.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481-504.

Jouany, J.P., 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments. 2, 131-144.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., Takashiba, S., 2003. Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. FEMS Immunol. Med. Mic. 39, 81-86.

McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agr. 48, 7-13.

McDonald, I., 1981. A revised model for the estimation of protein degradability in the rumen. J. Agric. Sci. Camb. 96, 251-256.

Mirzoeva, O.K., Grishanin, R.N., Calder, P.C., 1997. Antimicrobial action of propolis and some of its components: The effects on growth, membrane potential and motility of bacteria. Microbiol. Res. 152, 239-246.

Mould, F.L., Morgan, R., Kliem, K.E., Krystallidou, E., 2005. A review and simplification of the *in vitro* incubation medium. Anim. Feed Sci. Technol. 123-124, 155-172.

Nagaraja, T.G., Titgemeyer, E.C., 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. J. Dairy Sci. 90(Suppl. 1), E17-38.

Ørskov, E.R., Flatt, W.P., Moe, P.W., 1968. Fermentation Balance Approach to Estimate Extent of Fermentation and Efficiency of Volatile Fatty Acid Formation in Ruminants. J. dairy sci. 51, 1429-1435.

Oskoueian, E., Abdullah, N., Oskoueian, A., 2013. Effects of Flavonoids on Rumen Fermentation Activity, Methane Production, and Microbial Population. BioMed Res. Int. 2013, 8.

Ouwerkerk, D., Klieve, A.V., Forster, R.J., 2002. Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92, 753-758.

Øvreås, L., Torsvik, V., 1998. Microbial diversity and community structure in two different agricultural soil communities. Microb. Ecol. 36, 303-315.

Patra, A.K., Saxena, J., 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry, 71, 1198–1222.

Rhodes, M.J.C., 1996. Physiologically-active compounds in plant foods: An overview. P. Nutr. Soc. 55, 371-384.

Stack, R.J., Hungate, R.E., Opsahl, W.P., 1983. Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8. Appl. Environ. Microb. 46, 539-544.

Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y., 2001. Diet-Dependent Shifts in the Bacterial Population of the Rumen Revealed with Real-Time PCR. Appl. Environ. Microb. 67, 2766-2774.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48, 185-197.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Wang, D., Huang, J., Zhang, Z., Tian, X., Huang, H., Yu, Y., Zhang, G., Ding, J., Huang, R., 2013. Influences of Portulaca oleracea extracts on in vitro methane emissions and rumen fermentation of forage. J. Food Agric. Environ. 11, 483-488.

Winter, J., Moore, L.H., Dowell Jr, V.R., Bokkenheuser, V.D., 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microb. 55, 1203-1208.

Yaghoubi, S.M.J., Ghorbani, G.R., Rahmani, H.R., Nikkhah, A., 2007. In vitro manipulation of rumen fermentation by propolis flavonoids and monensin. J. Dairy Sci. 90(suppl. 1), 105-106.

Yáñez-Ruiz, D.R., MacÍas, B., Pinloche, E., Newbold, C.J., 2010. The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. FEMS Microbiol. Ecol. 72, 272-278.

CHAPTER 7

The Content of this Chapter is excluded from the text due to an agreement of confidentiality

CHAPTER 8
GENERAL DISCUSSION

Application of flavonoids could have impacts on both digestive microbial system (such as rumen environment) and animals' performance. In general, flavonoids can ameliorate the fermentation characteristics (Broudiscou et al., 2000) and can be served as anti-oxidant to decrease lipid oxidation and increase the quality of the meat production (Bodas et al. 2011). Besides, their effect on the surrounding environment via mitigating the enteric methane production is part of their advantages.

1- Effects of flavonoids on variations of rumen pH

From the results of our *in vivo* experiments, it can be demonstrated that flavonoids exert positive impacts on rumen pH, especially when the animals are reared under and intensive systems and fed high grains (concentrate) diets. In such conditions animals fed an excessive amount of readily fermentable carbohydrates and therefore increase the risk of acidotic conditions. To analyze the effect of Bioflavex® on acidotic environment, the acidosis was induced experimentally by administration of wheat grain (5 Kg) through the rumen canula. After administration of ground wheat, rumen pH decreased more in the CTR than in the treated heifers (Figure 1 Page 51). Changes in rumen pH are determined by fermentation products, such as VFA and lactic acid derived from carbohydrate fermentation (Owens et al., 1998). Lack of data on VFA and lactate concentrations while the lowest pH values were recorded make it unable to deduce if the concentrations of VFA and/or lactate were crucial in fluctuations of rumen pH. However, the higher lactate concentration (numerically) in CTR than in treated heifers after wheat supply may indicate that Bioflavex® supplementation prevented heifers from lactic acidosis.

Lactic acid is being produced during sub-acute acidosis; it does not accumulate because lactate-consuming bacteria remain active and rapidly metabolize it to VFA. However, as the pH reaches 5.0 or below for a sustained period, the growth of lactate-consuming bacteria is inhibited therefore lactate begins to accumulate. Thus, sub-acute acidosis has the potential to become lactic acidosis if the pH of 5.0 sustains for a concrete period of time (Nagaraja and Titgemeyer,

2007). In experiment 2, pH was also greater in Bioflavex® than in CTR heifers where the rumen concentration of VFA was also greater (Table 6 Page 49).

As mentioned previously, correlation between rumen VFA concentration and pH is low, and other mechanisms like saliva production related to a poor-roughage intake (Cummins et al., 2009) or type of starch sources (i.e corn or wheat grain) may have their impacts on rumen pH regulation. It is necessary to remark that in Exp. 2 heifers supplemented with Bioflavex® consumed more straw than CTR ones. Mechanisms whereby the supplementation with flavonoid may stimulate, straw consumption or modifying the activity of lactating-consuming bacteria probably need further investigation. In *in vitro* trials (Experiment 4, and 5) due to the presence of buffer solution in the incubation broth, the effect of flavonoid addition on rumen pH was negligible however in third experiment, a slight but significant improvement on rumen pH value was noticeable.

2- Effects of Bioflavex® and its pure flavonoids ingredients on the ruminal concentrations of VFA and their proportions.

Based on our *in vivo* and *in vitro* trials, supplementation of diet with Bioflavex® always affected volatile fatty acid composition towards desirable profile, increased VFA production and molar proportion of propionate at the expense of acetate. In the first *in vivo* experiment in which heifers were exposed to an experimental acidosis induction, flavonoids supplementation did not alter VFA concentration but reduced significantly the molar proportion of acetate (69.2 vs. 67.7) and improved the molar proportion of propionate although the differences in molar proportion of propionate between treated and CTR heifers did not reach statistical significance. In the growing trial (second experiment), where heifers during the growing period fed high concentrate diet plus straw *ad libitum*, the addition of Bioflavex® clearly showed its effects on improving both, i) rumen VFA concentration and ii) the molar proportion of propionate to the detriment of acetate, no changes were detected on the molar proportion of butyrate. Our hypothesis is that the Bioflavex® supplementation alters VFA proportion via changes in rumen fermentation, pushing the dynamic rumen environment ecosystem towards being more efficient, this issue was evidenced as the acetate/ propionate ratio tended to decrease (3.8 vs. 3.5) in heifers received Bioflavex®.

Moreover, a circadian variation in VFA proportion was detected which was buffered in Bioflavex® supplemented heifers, thus interaction between time (post feeding) and the presence

of Bioflavex® reached statistical significance (P<0.05). Throughout the days of study (49 to 70 d) Induced differences caused by Bioflavex® supplementation were gradually diminished (Figure 2 page 53). This trend suggests that rumen environment in growing heifers could be adapted in long period of time and diminished the impact of the presence of Bioflavex®, suggesting that the optimum effect of the citrus flavonoids blend could be expected in short administration periods.

In any case, reported *in vivo* values of rumen molar concentration of VFA or molar proportions of VFA are indicatives of the equilibrium of both production and absorption/pass processes of the end products, rather than its production ratio, which in an *in vitro* system this dynamicity cannot be expected. Effectively, in the fourth experiment using an *in vitro* system, flavonoid addition was able to improve rumen molar proportions of propionate and reduced the acetate-to-propionate ratio confirming previous findings registered in the *in vivo* assays. The *in vitro* system allows studying the kinetics of VFA synthesis without the bias induced by VFA absorption through the rumen wall.

The effect exerted by Bioflavex® on VFA profiles was a reflection of its ingredients, all the pure flavonoids substances in Bioflavex® complex reduced *in vitro* the molar proportion of acetate (Experiment 3 and 4) and (except Hesperidine in experiment 4) improved the molar proportion of propionate whereas in all cases acetate-to-propionate ratio was decreased.

The *in vitro* system confirms the activity of Bioflavex® on VFA fermentation profile and showed how this activity relies on its pure ingredients. These findings confirmed the fact that flavonoids exert some alteration in the rumen microbiota stimulating the growth of propionate-producing bacteria instead of acetate producing microorganism. Our results confirmed previous findings in the human gut, where genistein was able to modify prevalent species of microbiota (Clavel et al., 2005; Schoefer et al., 2002). Moreover, the effect of specific flavonoid compounds has also been demonstrated in specific rumen ecosystem, such as that of catequin on *Escherichia coli* (Tzounis et al., 2008) and *Bifidobacterium* (Gibson et al., 1995) or the Naringine on *Ruminococcus albus* (Stack et al., 1983) stimulated the growth of propionate-producing bacteria.

In our study, changes in the molar proportions of the major VFA were in agreement with changes in CH_4 production, thus, decreases in acetate-to-propionate ratio were reflected in a concomitant reduction of CH_4 production although this point would be further developed later, in the present discussion.

3- Effects of Bioflavex® and its pure flavonoids ingredients on gas and methane production.

Two *in vitro* trials (4 and 5) were designed to confirm the anti-microbial effect of the commercial flavonoids mixture and its ingredients on *in vitro* gas production, this more controlled *in vitro* assay may improve the understanding of Bioflavex® on rumen environment, and also allows identifying the effectiveness of the pure flavonoid ingredients in the Bioflavex® mixture. Effectively in both experiments it was observed that gas production was significantly reduced (Table 5 Page 75 and Table 3 Page 96). Gas production is an index of microbial fermentative activity, although it is true that changes in the molar proportion of VFA may cause small fluctuations on gas volume (Beuvink and Spoelstra, 1992). Therefore our results on gas production would confirm an unspecific impact against microbial fermentative activity of the flavonoid extracts from bitter orange and grapefruit. It is logical to expect that the effect of Bioflavex® on microbial environment should relay on the activity of its main flavonoid components in the mixture (i.e., NG, NH and PC). Effectively, NG and NH reduced microbial activity at a similar level (Table 5 Page 98). Moreover, the relationship between anti-microbial activity and the dose of flavonoids administration seem to indicate the existence of a threshold level or some type of synergic effect. Although Bioflavex® and all its pure compounds were added at the same level, so at different dosage, the observed reduction in gas production was equivalent in magnitude.

Anti-microbial activity of flavonoids on pathogenic (Wu et al., 2009) and nonpathogenic (Broudiscou and Lassalas, 2000) bacteria have been reported, and a hypothetical mechanism through the cleavage of C-ring in the flavonoids to produce the toxic phenolic acids has been described (Gladine et al., 2007c). The degradation of Naringine (as the main component of Bioflavex®) to the corresponding aglycone (Gladine et al., 2007c) and further C-ring cleavage to produce anti-microbial compounds (Winter et al., 1989) would explain some anti-microbial effect related to both Naringine and Bioflavex®.

Moreover evidences of the antimicrobial properties of Naringine has been provided by Mirzoeva et al. (1997) who described the antimicrobial activity of Naingine against *Escherichia coli* and its effect was mediated through the disruption of proton motive force and inhibition of bacterial motility. Besides, Naringine acts against the fibrolytic *Ruminococcus albus* (Stack et al., 1983).

The differential activity of flavonoids structures on *in vitro* rumen microbial fermentation has been previously described by (Broudiscou and Lassalas, 2000) and the structure-activity relationship has been widely documented by Cushnie and Lamb (2011).

In the fifth in *vitro* trial it was observed that concomitant with the reduction in volume of the produced gas, the toxic CH_4 production and its proportion was also reduced. Methane production was mitigated with Bioflavex® and its major pure flavonoids (Neohesperidine and Poncirine) whereas Bioflavex® and Poncirine reduced the concentration of CH_4 inside the produced gas (Table 3 Page 96). Somehow, this fact would suggest a specific inhibition of flavonoid substances on methanogens that would differ among flavonoids types. The mitigating effect of flavonoids on methane production has been suggested 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. 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).

Previous reports showed 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 (Bodas et al., 2012; Broudiscou and Lassalas, 2000). In the light of this hypothesis, we demonstrated that flavonoids also decreased acetic acid concentration and thus its availability for those organisms that use acetic acid for methanogenesis. Using *Methanosarcina* as acetoclastic methanogenic archaea´s flag, we cannot detect any significant increase in acetoclastic population; on the contrary, flavonoids substances, to some degree, exerted a depression in its population, which would confirm a direct toxicity of flavonoids on methanogenic population.

Alternatively, Garcia-Lopez et al. (1996) have suggested that flavonoids may act directly as electron acceptors, although if so there would be a direct relationship between the doses or levels of flavonoids in the media and the level of hydrogen availability and the further methane inhibition.

4- Regulatory effect of Bioflavex® on rumen microbial population.

At the rumen level each fermentative process which happens is due to its microbes. Based on *in vivo* and *in vitro* experiments, it can be seen that Bioflavex® and its pure flavonoid substances have their effects on rumen inhabitant microbes in terms of absolute or relative abundances.

4.1. *Microbial population*

As it was mentioned above, improvements on pH level, concentration of VFAs and mitigation of methane production have been yielded using Bioflavex® and/or its pure flavonoid components, but the critical point is that the flavonoids do all these improvements via regulating the microbial phase and their interactions. As an explanation of these alterations in abundances of bacteria (regulation) it should be mentioned that flavonoids are able to alter the bioenergetic status of the bacterial membrane (Mirzoeva *et al*., 1997) or they can degrade to such metabolites as phenolic acid or 3, 4-dihydroxyphenilaacetic with anti-microbial properties (Schneider *et al*., 1999; Winter *et al*., 1989).

4.2. *Lactate producing-consuming bacteria*

The relationship between VFA, pH and acidosis was studied previously and in relation to lactate, when its concentrations exceed 50 m*M* during acute acidosis, rumen pH can decline between 3.9 and 4.5 (Nagaraja et al., 1985); however, other studies detected slight increases in total rumen lactate concentrations [e.g., <10 (Burrin and Britton, 1986) 5 (Goad et al., 1998) or virtually no increases at all; e.g., <0.4 m*M* (Coe et al., 1999; Bevans et al., 2005)] under sub-acute situations. In the first experiment, our results showed a significant accumulation of lactate (L+ plus D-) occurred after acidosis was experimentally induced, although that increase never exceeded 1.5 m*M* or 122.5 mg/l (Table 4, Page 47).

Lactic acid accumulates when the amount of starch and other sources of readily degradable carbohydrates increase suddenly in the rumen, which stimulates the proliferation of rapidly growing lactic acid-producing bacteria, (e.g., *S. bovis*) to the point where the growth of lactic-producing microorganisms exceeds the growth rate of lactic acid-utilizing bacteria (Russell and Hino, 1985); consequently, lactic acid accumulates. At this point, it is necessary to remark that under rumen acidic condition lactate absorption through the rumen wall reaches at its minimum level (Owens et al., 1998). In our first experiment (*in vivo*), it is shown that after applying the wheat supplement through rumen cannula, there was an increase in rumen concentrations of lactate and the ruminal abundances of *M. elsdenii* were higher in the treated than in the CTR

heifers, which might have been associated with the mechanisms whereby flavonoids modulate rumen pH. Moreover, *S. Bovis* the main lactate-producing specie increased and the maximum abundances coincided with the highest lactate concentrations, however, it is likely that the simultaneous increase in lactate-consuming bacteria (i.e *S. ruminantium* and *M. elsdenii*) might have mitigated the lactate accumulation. Existing literature evidenced that after acidosis induction (during an adaptation to high-concentrate diet), only a short-term increase in abundances of lactate-consuming bacteria can be observed (Goad et al., 1998; Tajima et al., 2001) because most lactate-consuming bacteria cannot tolerate low pH conditions (Russell and Hino, 1985), that was not our case.

The third experiment also provides evidences that lactate concentration was reduced under effect of applying the Bioflavex® in the diet and again such reduction was concomitant with the improvement in abundances of lactate-consuming bacteria (*M. elsdenii* and *S. ruminantium*).

Data analysis of the *in vitro* trial (experiment 4), showed that no treatment effect was observed on the concentration of lactate (probably due to lack of dynamicity inside the bottles -Non-published results) however, abundances of lactate-consuming species were improved confirming values registered in the *in vivo* trials whereas the abundance of *S. bovis* (lactate-producing bacteria) was diminished with the presence of flavonoids in the incubation solution.

The promotion of lactate-consuming bacteria exerted by Bioflavex® definitively relies on the activity of Naringine and Neohespiridine as the main components of the citrus blend. No effect of Naringine and Neohespiridine were observed on *S. ruminantium* but the presence of Neohespiridine (and apparently Naringine) improved significantly the abundance of *M. elsdenii*, but Neohespiridine also exerted a significant depression on *S. bovis* (Table 7 Page 77). Pure flavonoid substances showed clearer effect than the commercial mixture (Bioflavex®) although such activity was not seen in all tested flavonoids. Neoeriocitrine did not exert positive effect on lactate-consuming bacteria and on the contrary promoted the growth of *S. bovis*.

Therefore, flavonoids compounds seem to have antimicrobial properties but the mechanism may be different and depends on their chemical nature.

4.3. *Methanogen population*

Dietary addition of Bioflavex® induced significant changes in volatile fatty acids (VFA) production and its profile which may imply on a di-hydrogen re-canalization and thus changes in CH_4 synthesis (Demeyer and Van Nevel, 1975), assuming that in ruminant species CH_4 is the

major sink of the excess of di-hydrogen released during VFA synthesis and CH_4 production negatively correlated with energy utilization and microbial mass synthesis on ruminants (Ørskov et al., 1968; Yáñez-Ruiz et al., 2010).

In the fifth *in vitro* experiment the addition of Bioflavex® reduced CH_4 production (mL/ g DM) and its proportion inside the produced gas (mL/ mL) at 12 h post incubation period. The impact of Bioflavex® on VFA production had minor relevance, but depressed CH_4 production and increased the proportion of propionate instead of acetate. Our results agree well with Wang et al. (2013), who reported a significant depression of methanogens population using an extract of the plant *Portulaca oleracea*, rich in flavonoids.

Methane synthesized in the rumen environment come from the activity of HMA population, and again data showed in Table 5 (Page 98) reveal how the specific activity of Bioflavex® may rely on its principal components (NG and NH), that both depressed significantly the abundance of HMA and methanosarcina *spp*. A close relationship between HMA abundance (Log of *mcrA* gene copies number/g FM) and the CH_4 synthesis in bottles supplemented with Bioflavex® was observed. The *in vitro* CH_4 inhibitory activity of Naringine (also quercetin) was firstly demonstrated by Oskoueian et al. (2013), as well as other polyphenol compounds like 9,10-anthraquinone by García-Lopez et al. (1996) and complex of plant mixtures rich in flavonoids by Broudiscou et al. (2000).

5- Effect of Bioflavex® on growth performance

In general, the performance (1.1 SEM 0.06; ADG) of the finishing heifers in the second experiment was lower than the values (1.36) proposed in the Meta analyses by Zinn *et al.* (2008) for growing and finishing heifers and also than the other studies related to finishing heifers (1.0 to 1.24; Bindel et al., 2000). The inclusion of Bioflavex® improved numerically ADG but not DM intake, thus feed conversion ratio was also high in animals fed Bioflavex®. Lack of differences in ADG may be partially attributed to, i) the low ADG which may mask the potential effect of the flavonoid compound; ii) the high level of residual variation (SEM 0.06) recorded in the present assay in comparison with trials using similar experimental protocol (Bindel et al., 2000; Devant et al., 2007; González et al., 2008). Few data are available on the effect of flavonoids blend on the animal performance. De Freitas *et al*., (2007) fed propolis as flavonoids source to daily cows demonstrated a positive effect on milk yield, although Devant *et al.* (2007) used different plant extracts rich in flavonoids and were not able to demonstrate any

improvement on feedlot performance of Holstein bulls in terms of growth rate or Growth: Feed ratio. Devant *et al.* (2007) mentioned that the plant extracts mixture is usually composed of several tertiary compounds species such as sarsaponin, a steroidal saponin and the potential existence of collateral effects among tertiary compounds should be not neglected (Broudiscou and Lassalas, 2000).

Heifers fed high concentrate diet [up to 90 %, table 4] complemented with low quality roughage such as barley straw. Despite that animals did not showed apparent signs of acidosis, the average rumen pH value (6.59, 6.07 and 6.11 at 2, 4 and 8 h post feeding, respectively) were in the range (6.16 and 5.71) of daily pH values for cattle consuming concentrate-based diets (Beauchemin et al., 2001; Koenig et al., 2003). Therefore, the daily pH level was upper than the pH value limit (5.5) considered as acidotic threshold (Cooper and Klopfenstein, 1996) but throughout the trial around 30 % of cannulated heifers, at 2h post feeding showed acidotic levels (pH< 5.6). Indeed this incidence might negatively affect the status of the rumen environment, DMI and consequently ADG.

Acidosis is related to an excessive intake of readily fermentable carbohydrates, although the rate of starch cleavage to glucose varies among grain source. Certain starch sources (i.e corn or milo grain) are embedded in a protein matrix and thus starch granules have less surface exposed for microbial attack. Then delayed fermentation of starch in corn would alleviate the propensity of acidosis, as in our study. In this sense rumen VFA concentration (m*M*) varied from 60 to 85, (69.9 SEM 0.175) agreed well with those values reported in finishing heifers in corn-based diets (Devant et al., 2007;) but were much lower than those which fed barley grain (Ghorbani et al., 2002; Koenig et al., 2003).

In Exp. 2 The VFA concentration at each sampling time were in the range of other studies (Koenig et al., 2003) and followed the same pattern, recording the lowest values was before (0 h) and the highest values at 2 and 4 h post feeding, and it may reflect in the multiple meals that the heifers consumed during the day. Probably VFA concentration would have been much higher at 2 or 4 hours if animals had been restricted to a single meal. In this study circadian variation in pH seems to be inversely associated with the variation in VFA (Table 6 Page 49) however, this relationship was not always true and flavonoids supplementation induced both, high level of VFA together with the highest pH values in comparison with the control group. Other substances can exert a significant effect on the rumen pH. Among them ammonia, however, in the feeding

trial flavonoids supplementation reduced consistently rumen ammonia concentration but pH level rose.

Despite all improvements in rumen fermentation (Exp.2 and 4; pH, molar proportion of propionate and urine PD excretion), there was no apparent improvement in performance and efficiency. Limited information is available on the effects of mixtures of flavonoids on animal performance. Rumen molar proportions of propionate were enhanced in the heifers that fed flavonoids. Tissues use propionate more efficiently than they use acetate. In addition to direct oxidation through the citric acid cycle, propionate has the potential to be used in gluconeogenesis. However, it is likely that a more efficient use of propionate has a small effect on animal performance (Schelling, 1984). Furthermore, total rumen molar production and absorption of propionate are important factors in assessing the impact of propionate on tissue energetics, but rumen concentrations and molar proportions of propionate only indicate the equilibrium between the production and absorption of the end products, rather than its absorption.

In Exp 2, an improvement in the duodenal flow of microbial protein (a protein source that has a good amino acid balance) was reflected in the urine PD excretion of the treated heifers, but it did not improve heifers' ADG (Table 6 Page 49). In beef cattles fed high-concentrate diet, up to 45% of dietary protein was found in total duodenal N flow (Devant et al., 2001; Martín-Orúe et al., 1998) thus, an improvement in rumen microbial yield might have a small effect on total AA flow and, consequently, on animal growth.

Discussion on the chapter 7th of the thesis is excluded from this section due to an agreement of confidentiality.

CHAPTER 9
CONCLUSIONS

Based on the obtained data from the *in vivo* assays (Exp. 1, 2, 3 and 6) it can be concluded that:

1- The addition (300 mg/Kg) of the commercial mixture of citrus extract known as Bioflavex® mitigate the acidotic effect of induced acidosis by wheat grain engorgement. Bioflavex® buffered the decrease in pH and reduced the time in which pH was below the acidotic level.

2- In the growing trial, heifers were reared under and intensive growing system fed high concentrate diet, the addition of (300 mg/Kg DM) Bioflavex® to the concentrate consistently affected the rumen fermentation at two stages, first, reduced the incidence of rumen acidosis derive from high concentrate intake and second, improved the volatile fatty acid concentration ameliorating molar proportion of propionate in detriment of acetate.

3- Throughout the growing trial, differences induced by experimental treatment were progressively diminished; it may suggest an adaptation of rumen ecosystem to the presence of flavonoids in the media.

4- The addition of Bioflavex® under the acidosis induction improved significantly the abundances of lactate-consuming species (*M. elsdenii*) and did not alter those of lactate-producing ones.

5- Despite the positive effects of flavonoid supplementation on rumen pH, VFA proportions and microbial yield no significant effects on performance were detected.

Based on the obtained data from the *in vitro* trials (Exp. 4 & 5) it can be concluded that:

1- Using an "*in vitro*" system where rumen liquor was incubated under conditions simulating the rumen environment in animals that were fed high concentrate rations, Bioflavex® and its pure flavonoids components modified gas production, the addition of Neohespiridine, Bioflavex®, Isonaringine, and Hespiridine to the media reduced gas production whereas the effect of Poncirine Neoeriocitrine cannot be distinguished from the control.

2- After twelve hours post incubation, the presence of flavonoids in the simulated ruminal environment altered methane production, Bioflavex®, Neohespiridine, Poncirine, Isonaringine and

Hesperidine mitigated its synthesis whereas the effect of Naringine and Neoeriocitrine cannot be distinguished from the control.

3- After twelve hours post incubation, the presence of flavonoid in the culture media did not alter ammonia or VFA concentration but clearly improved propionate synthesis in detriment of acetate.

4- Relative abundances ($2^{-\Delta Ct}$) of *Methanosarcina Spp.* and hydrogenotrophic methanogenic archaea were reduced by the addition of Bioflavex® in the media, the effect of Bioflavex® can be explained by the individual effect of its main components.

5- Bioflavex® did not alter the relative abundance of *S. bovis* (lactate-producing bacteria) while promote that of *M. elsdenii* (lactate-consuming bacteria). The role of Bioflavex® clearly relied on its main components (Naringine, Neohesperidine and Poncirine) that showed a similar activity, whereas Neoeriocitrine showed the reversed effects in relation to Bioflavex® and its main components.

Conclusions on the chapter 7th of the thesis are excluded from this section due to an agreement of confidentiality.

REFERENCES

Acamovic, T., and J. D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. Proceedings of the Nutrition Society 64: 403-412.

AFRC. 1993. Energy and Protein Requirements of Ruminants. CAB International, Wallingford, U.K.

Ahn, J., I. U. Grün, and L. N. Fernando. 2002. Antioxidant properties of natural plant extracts containing polyphenolic compounds in cooked ground beef. Journal of Food Science 67: 1364-1369.

Akin, D. E. 1986. Chemical and biological structure in plants as related to microbial degradation of forage cell walls. In: L. P. Milligan, W. L. Grovum and A. Dobson (eds.) Control of Digestion and Metabolism in Ruminants. p 139-157. Prentice Hall, Englewood Cliffs, New Jersey.

Allard, J. P., R. Kurian, E. Aghdassi, R. Muggli, and D. Royall. 1997. Lipid peroxidation during n-3 fatty acid and vitamin E supplementation in humans. Lipids 32: 535-541.

Arts, I. C. W., P. C. H. Hollman, and D. Kromhout. 1999. Chocolate as a source of tea flavonoids. Lancet 354: 488.

Arts, I. C. W., B. Van De Putte, and P. C. H. Hollman. 2000. Catechin contents of foods commonly consumed in The Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. Journal of Agricultural and Food Chemistry 48: 1746-1751.

Aureli, P., A. Costantini, and S. Zolea. 1992. Antimicrobial activity of some plant essential oils against Listeria monocytogenes. J. Food Prot. 55: 344-348.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. Journal of dairy science. 88 Suppl 1: E9-21.

Baker, S. K. 1999. Biology of rumen methanogens, and the stimulation of animal immunity. Meeting the Kyoto Target. Implications for the Australian Livestock Industries: 41-49.

Balcells, J., Aris, A., Serrano, A., Seradj, A.R., Crespo, J., Devant, M., 2012. Effects of an extract of plant flavonoids (bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. J. Anim. Sci. 90, 4975-4984.

Beauchemin, K. A., and J. G. Buchanan-Smith. 1990. Effects of Fiber Source and Method of Feeding on Chewing Activities, Digestive Function, and Productivity of Dairy Cows. J. Dairy Sci. 73: 749-762.

Beauchemin, K. A., M. Kreuzer, F. O'Mara, and T. A. McAllister. 2008. Nutritional management for enteric methane abatement: A review. Australian Journal of Experimental Agriculture 48: 21-27.

Beauchemin, K. A., T. A. McAllister, and S. M. McGinn. 2009. Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 4: 1-18.

Beauchemin, K. A., and S. M. McGinn. 2005. Methane emissions from feedlot cattle fed barley or corn diets. Journal of Animal Science 83: 653-661.

Beauchemin, K.A., Yang, W.Z., Rode, L.M., 2001. Effects of barley grain processing on the site and extent of digestion of beef feedlot finishing diets. Journal of animal science 79, 1925-1936.

Benchaar, C., Chaves, A.V., Fraser, G.R., Wang, Y., Beauchemin, K.A., McAllister, T.A., 2007. Effects of essential oils and their components on in vitro rumen microbial fermentation. Canadian Journal of Animal Science 87, 413-419.

Beuvink, J.M.V., Spoelstra, S.F., 1992. Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro. Applied Microbiology and Biotechnology 37, 505-509.

Bevans, D. W., K. A. Beauchemin, K. S. Schwartzkopf-Genswein, J. J. McKinnon, and T. A. McAllister. 2005. Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle. J. Anim. Sci. 83: 1116-1132.

Bindel, D.J., Drouillard, J.S., Titgemeyer, E.C., Wessels, R.H., Löest, C.A., 2000. Effects of ruminally protected choline and dietary fat on performance and blood metabolites of finishing heifers. J. Anim. Sci. 78, 2497-2503.

Blaxter, K. L., and J. L. Clapperton. 1965. Prediction of the amount of methane produced by ruminants. British Journal of Nutrition 19: 511-522.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F.J., López, S., 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Tech. 176, 78-93.

Bodas, R., N. Prieto, O. López-Campos, F. J. Giráldez, and S. Andrés. 2011. Naringin and vitamin E influence the oxidative stability and lipid profile of plasma in lambs fed fish oil. Research in Veterinary Science 91: 98-102.

Brannan, R. G., and E. Mah. 2007. Grape seed extract inhibits lipid oxidation in muscle from different species during refrigerated and frozen storage and oxidation catalyzed by peroxynitrite and iron/ascorbate in a pyrogallol red model system. Meat Science 77: 540-546.

Brock, F. M., C. W. Forsberg, and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Applied and Environmental Microbiology 44: 561-569.

Broudiscou, L.-P., Y. Papon, and A. F. Broudiscou. 2000. Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes. Anim. Feed Sci. Tech. 87: 263-277.

Broudiscou, L. P., and B. Lassalas. 2000. Effects of Lavandula officinalis and Equisetum arvense dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40: 431-440.

Broudiscou, L. P., Y. Papon, and A. Broudiscou. 1999. Effects of inorganic nitrogen and amino acids on the degradation of ammonia-treated barley straw and proteosynthesis in a continuous culture of rumen microbes. Anim. Feed Sci. Tech. 77: 149-162.

Brouwer, E. 1965. Report of sub-committee on constants and factors. In: K. L. Blaxter (ed.) Energy Metabolism. p 441-443. Academic Press London, UK.

Burrin, D. G., and R. A. Britton. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63: 888-893.

Burt, S. 2004. Essential oils: Their antibacterial properties and potential applications in foods - A review. International Journal of Food Microbiology 94: 223-253.

Cambra-López, M., P. García Rebollar, F. Estellés, and A. Torres. 2008. Estimación de las emisiones de los rumiantes en España: el factor de conversión de metano. Arch. Zootec. 57: 89-101.

Campo, M.M., Nute, G.R., Hughes, S.I., Enser, M., Wood, J.D., Richardson, R.I., 2006. Flavour perception of oxidation in beef. Meat Science 72, 303-311.

Cao, G., E. Sofic, and R. L. Prior. 1997. Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. Free Radical Biology and Medicine 22: 749-760.

Cao, Z., X. Di Liao, J. B. Liang, Y. B. Wu, and B. Yu. 2012. Diversity of methanogens in the hindgut of grower and finisher pigs. Afr. J. Biotechnol. 11: 4949-4955.

Cardozo, P., S. Calsamiglia, and A. Ferret. 2002. Effects of pH on nutrient digestion and microbial fermentation in a dual flow continuous culture system fed a high concentrate diet. J. Dairy Sci (Suppl. 1) 85: 182.

Castrillo, C., and J. Balcells. 2002. Aspectos básicos del metabolismo ruminal del nitrógeno. In: Pequeños Rumiantes. p 14-26.

Chen, M., and M. J. Wolin. 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microb. 38: 72-77.

Cheng, I. F., and K. Breen. 2000. On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the fenton reaction of the iron-ATP complex. BioMetals 13: 77-83.

Chiriase, N. K., D. P. Hutcheson, and G. B. Thompson. 1991. Feeding behavior of steers and heifers fed high concentrate diet with or without monensin. J. Anim. Sci. 69: 61.

Chouliara, I., and M. G. Kontominas. 2006. Combined effect of thyme essential oil and modified atmosphere packaging to extend shelf life of fresh chicken meat. In: J. N. Govil, V. K. Singh, K. Almad and R. K. Sharma (eds.) Recent Progress in Medicinal Plants: Natural Products No. 15. p 423-442. Studium Press LLC, Houston, TX.

Clavel, T., Henderson, G., Alpert, C.A., Philippe, C., Rigottier-Gois, L., Doré, J., Blaut, M., 2005. Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. Appl. Environ. Microb. 71, 6077-6085.

Coe, M.L., Nagaraja, T.G., Sun, Y.D., Wallace, N., Towne, E.G., Kemp, K.E., Hutcheson, J.P., 1999. Effect of Virginiamycin on Ruminal Fermentation in Cattle during Adaptation to a High Concentrate Diet and during an Induced Acidosis. J. Anim. Sci. 77, 2259-2268.

Cooper, R., Klopfenstein, T., 1996. Effect of rumensin and feed intake variation on ruminal pH, Scientific Update on Rumensin/tylan/micotil for the Professional Feedlot Consultant, Elanco Animal Health, Indianapolis, IN, p. A1.

Cooper, R., T. Klopfenstein, R. Stock, and C. Parrott. 1998. Observations on acidosis through continual feed intake and ruminal pH monitoring. Nebraska Beef Rep.: 75.

Cotta, M. A. 1988. Amylolytic activity of selected species of ruminal bacteria. Applied and Environmental Microbiology 54: 772-776.

Cotta, M. A. 1992. Interaction of ruminal bacteria in the production and utilization of maltooligosaccharides from starch. Applied and Environmental Microbiology 58: 48-54.

Cotta, M. A., and J. B. Russell. 1997. Digestion in the rumen: A model for metabolism of nitrogen compounds in gastrointestinal environments. In: R. I. Mackie and B. A. White (eds.) Gastrointestinal Microbiology No. 1. p 380-423. Chapman & Hall, New York.

Cottle, D. J., J. V. Nolan, and S. G. Wiedemann. 2011. Ruminant enteric methane mitigation: A review. Animal Production Science 51: 491-514.

Crichlow, E. C., and R. K. Chaplin. 1985. Ruminal lactic acidosis: relationship of forestomach motility to nondissociated volatile fatty acids levels. American Journal of Veterinary Research 46: 1908-1911.

Croft, K. D. 1998. The chemistry and biological effects of flavonoids and phenolic acids Annals of the New York Academy of Sciences No. 854. p 435-442.

Cummins, B., O'Kiely, P., Keane, M.G., Kenny, D.A., 2009. Feed intake pattern, behaviour, rumen characteristics and blood metabolites of finishing beef steers offered total mixed rations constituted at feeding or ensiling. Ir. J. Agric. Food Res. 48, 57-73.

Cushnie, T. P. T., and A. J. Lamb. 2011. Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Ag. 38: 99-107.

Czerkawski, J. W. 1969. Methane production in ruminants and its significance. World review of nutrition and dietetics 11: 240-282.

De Freitas, J.A., De Souza, J.C., Lana, R.P., Antonangelo, R.P., De Freitas, A.A., De Santana, R.T.S., 2007. Ethanolic extract of propolis in lactating cows. J. Dairy sci. 90(suppl. 1), 105.

Demeyer, D. I., and C. J. Van Nevel. 1975. Methanogenesis, an integrated part of carbohydrate fermentation, and its control Digestion and Metabolism in the Ruminant. p 366-382.

Dennis, S. M., T. G. Nagaraja, and E. E. Bartley. 1981. Effects of lasalocid or monensin on lactate-producing or -using rumen bacteria. Journal of Animal Science 52: 418-426.

Depardon, N., D. Debroas, and G. Blanchart. 1996. Breakdown of peptides from a casein hydrolysate by rumen bacteria. Simultaneous study of enzyme activities and physicochemical parameters. Reproduction Nutrition Development 36: 457-466.

Devant, M., A. Anglada, and A. Bach. 2007. Effects of plant extract supplementation on rumen fermentation and metabolism in young Holstein bulls consuming high levels of concentrate. Anim. Feed Sci. Tech. 137: 46-57.

Devant, M., Ferret, A., Calsamiglia, S., Casals, R., Gasa, J., 2001. Effect of nitrogen source in high-concentrate, low-protein beef cattle diets on microbial fermentation studied in vivo and in vitro. J. Anim. Sci. 79, 1944-1953.

Dijkstra, J. 1994. Simulation of the dynamics of protozoa in the rumen. British Journal of Nutrition 72: 679-699.

Eckard, R. J., C. Grainger, and C. A. M. de Klein. 2010. Options for the abatement of methane and nitrous oxide from ruminant production: A review. Livestock Science 130: 47-56.

Eugène, M., D. Sauvant, J. L. Weisbecker, and H. Archimède. 2010. Effects of defaunation on digestion of fresh digitaria decumbens grass and growth of lambs. Animal 4: 439-445.

European Communities. 2003. Regulation EC No 1831/2003 of the European Parliament and Council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur. Union L268: 29-43.

Faichney, G. J., N. M. Graham, and D. M. Walker. 1999. Rumen characteristics, methane emissions, and digestion in weaned lambs reared in isolation. Australian Journal of Agricultural Research 50: 1083-1089.

Feng, P., C. W. Hunt, G. T. Pritchard, and S. M. Parish. 1995. Effect of barley variety and dietary barley content on digestive function in beef steers fed grass hay-based diets. Journal of Animal Science 73: 3476-3484.

Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., Comporti, M., 1997. Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. FEBS Letters 416, 123-129.

Firkins, J. L., and C. Reynolds. 2005. Whole-animal nitrogen balance in cattle. In: A. N. Hristov and E. Pfeffer (eds.) Nitrogen and Phosphorus Nutrition of Cattle: Reducing the Environmental Impact of Cattle Operations. p 167-186. CABI International.

Flint, H. J., and C. W. Forsberg. 1995. Polysaccharide degradation in the rumen: Biochemistry and genetics. In: W. V. Englehardt, S. Leonard-Marek, G. Breves and D. Giesecke (eds.) Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. p 43-70. Ferdinand Enke Verlag, Stuttgart.

Forsberg, C. W., E. Forano, and A. Chesson. 2000. Microbial adherence to the plant cell wall and enzymatic hydrolysis. In: P. B. Cronje (ed.) Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. p 79-97. CABI Publishing, Pretoria, South Africa.

France, J., and R. C. Siddons. 1993. Volatile fatty acid production. In: J. M. Forbes and J. France (eds.) Quantitative Aspects of Ruminant Digestion and Metabolism. p 107-121. CAB International, New York.

Fraser, G.R., Chaves, A.V., Wang, Y., McAllister, T.A., Beauchemin, K.A., Benchaart, C., 2007. Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems. Journal of Dairy Science 90, 2315-2328.

Gadang, V. P., N. S. Hettiarachchy, M. G. Johnson, and C. Owens. 2008. Evaluation of antibacterial activity of whey protein isolate coating incorporated with nisin, grape seed extract, malic acid, and EDTA on a turkey frankfurter system. Journal of Food Science 73: M389-M394.

Galati, G., and P. J. O'Brien. 2004. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. Free Radical Biology and Medicine 37: 287-303.

Garcia-Lopez, P. M., L. Kung, and J. M. Odom. 1996. In vitro inhibition of microbial methane production by 9,10-anthraquinone. J. Anim. Sci. 74: 2276-2284.

Ghorbani, G.R., Morgavi, D.P., Beauchemin, K.A., Leedle, J.A.Z., 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. J. Anim. Sci. 80, 1977-1985.

Giannenas, I., Florou-Paneri, P., Papazahariadou, M., Christaki, E., Botsoglou, N.A., Spais, A.B., 2003. Effect of dietary supplementation with oregano essential oil on performance of broilers after experimental infection with Eimeria tenella. Archives of Animal Nutrition 57, 99-106.

Gibson, G.R., Beatty, E.R., Wang, X., Cummings, J.H., 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108, 975-982.

Gilbert, H. J., G. P. Hazlewood, J. I. Laurie, C. G. Orpin, and G. P. Xue. 1992. Homologous catalytic domains in a rumen fungal xylanase: Evidence for gene duplication and prokaryotic origin. Molecular Microbiology 6: 2065-2072.

Gladine, C., C. Morand, E. Rock, D. Bauchart, and D. Durand. 2007a. Plant extracts rich in polyphenols (PERP) are efficient antioxidants to prevent lipoperoxidation in plasma lipids

from animals fed n - 3 PUFA supplemented diets. Animal Feed Science and Technology 136: 281-296.

Gladine, C., Morand, C., Rock, E., Gruffat, D., Bauchart, D., Durand, D., 2007b. The antioxidative effect of plant extracts rich in polyphenols differs between liver and muscle tissues in rats fed n-3 PUFA rich diets. Animal Feed Science and Technology 139, 257-272.

Gladine, C., E. Rock, C. Morand, D. Bauchart, and D. Durand. 2007c. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Brit. J. Nutr. 98: 691-701.

Goad, D. W., C. L. Goad, and T. G. Nagaraja. 1998. Ruminal Microbial and Fermentative Changes Associated with Experimentally Induced Subacute Acidosis in Steers. J. Anim. Sci. 76: 234-241.

Grant, R. J. 1997. Interactions among Forages and Nonforage Fiber Sources. Journal of Dairy Science 80: 1438-1446.

Greathead, H. 2003. Plants and plant extracts for improving animal productivity. Proceedings of the Nutrition Society 62: 279-290.

Hamilton-Miller, J. M. T. 1995. Antimicrobial properties of tea (Camellia sinensis L.). Antimicrobial Agents and Chemotherapy 39: 2375-2377.

Hao, Y. Y., R. E. Brackett, and M. P. Doyle. 1998. Inhibition of Listeria monocytogenes and Aeromonas hydrophila by plant extracts in refrigerated cooked beef. Journal of Food Protection 61: 307-312.

Haraguchi, H., K. Tanimoto, Y. Tamura, K. Mizutani, and T. Kinoshita. 1998. Mode of antibacterial action of retrochalcones from Glycyrrhiza inflata. Phytochemistry 48: 125-129.

Harborne, J. B., and C. A. Williams. 2000. Advances in flavonoid research since 1992. Phytochemistry 55: 481-504.

Hartmann, T. 2007. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. Phytochemistry 68: 2831-2846.

Havsteen, B. H. 2002. The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics 96: 67-202.

Hegarty, R. S. 1999. Reducing rumen methane emissions through elimination of rumen protozoa. Australian Journal of Agricultural Research 50: 1321-1327.

Heim, K. E., A. R. Tagliaferro, and D. J. Bobilya. 2002. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. Journal of Nutritional Biochemistry 13: 572-584.

Hertog, M. G. L. 1992. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. Journal of Agricultural and Food Chemistry 40: 2379-2383.

Hertog, M. G. L., P. C. H. Hollman, M. B. Katan, and D. Kromhout. 1993. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutrition and Cancer 20: 21-29.

Hobson, P. N., and C. S. Stewart. 1997. The Rumen Microbial Ecosystem. Chapman & Hall, New York.

Höglund-Isaksson, L. 2012. Global anthropogenic methane emissions 2005-2030: Technical mitigation potentials and costs. Atmospheric Chemistry and Physics 12: 9079-9096.

Hollman, P. C. H. 2004. Absorption, bioavailability, and metabolism of flavonoids. Pharmaceutical Biology 42: 74-83.

Houghton, J. T. et al. 1996. IPCC Revised 1996 guidelines for national greenhouse gas inventories, Reference manual, Chapter 4- Agriculture. IPCC, Bracknell.

Hungate, R. E. 1966. The Rumen and Its Microbes. Academic Press, New York-London.

Huntington, G. B. 1997. Starch Utilization by Ruminants: From Basics to the Bunk. Journal of Animal Science 75: 852-867.

Huntington, G. B., E. J. Zetina, J. M. Whitt, and W. Potts. 1996. Effects of Dietary Concentrate Level on Nutrient Absorption, Liver Metabolism, and Urea Kinetics of Beef Steers Fed Isonitrogenous and Isoenergetic Diets. Journal of Animal Science 74: 908-916.

Ikigai, H., T. Nakae, Y. Hara, and T. Shimamura. 1993. Bactericidal catechins damage the lipid bilayer. Biochimica et Biophysica Acta - Biomembranes 1147: 132-136.

INRA. 1989. Ruminant Nutrition. Recommended Allowances and Feed Tables. INRA and John Libbey Eurotext, Paris.

Jagetia, G. C., and T. K. Reddy. 2011. Alleviation of iron induced oxidative stress by the grape fruit flavanone naringin in vitro. Chemico-Biological Interactions 190: 121-128.

Jeon, S.M., Bok, S.H., Jang, M.K., Kim, Y.H., Nam, K.T., Jeong, T.S., Park, Y.B., Choi, M.S., 2002. Comparison of antioxidant effects of naringin and probucol in cholesterol-fed rabbits. Clinica Chimica Acta 317, 181-190.

Jeon, S.M., Bok, S.H., Jang, M.K., Lee, M.K., Nam, K.T., Park, Y.B., Rhee, S.J., Choi, M.S., 2001. Antioxidative activity of naringin and lovastatin in high cholesterol-fed rabbits. Life Sciences 69, 2855-2866.

Johnson, D. E., H. W. Phetteplace, and A. F. Seidl. 2002. Methane, nitrous oxide and carbon dioxide emissions from ruminant livestock production systems. In: GHGes and Animal Agriculture, Obihiro, Japan. p 77-85.

Johnson, D. E., G. M. Ward, and J. J. Ramsey. 1996. Livestock methane: Current emissions and mitigation potential. In: E. T. Kornegay (ed.) Nutrient Management of Food Animals to Enhance and Protect the Environment. p 219-234. Lewis Publishers, New York.

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. Journal of Animal Science 73: 2483-2492.

Jones, R. J., and J. B. Lowry. 1984. Australian goats detoxify the goitrogen 3-hydroxy-4(1H) pyridone (DHP) after rumen infusion from an indonesian goat. Experientia 40: 1435-1436.

Jørgensen, H., P. K. Theil, and K. E. B. Knudsen. 2011. Enteric methane emission from pigs. In: E. Carayannis (ed.) Planet Earth - Global warming challenges and opportunities for policy and practice. p 605-622. InTech.

Jouany, J. P., and D. P. Morgavi. 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. Animal 1: 1443-1466.

Kannan, G., Saker, K.E., Terrill, T.H., Kouakou, B., Galipalli, S., Gelaye, S., 2007. Effect of seaweed extract supplementation in goats exposed to simulated preslaughter stress. Small Ruminant Research 73, 221-227.

Kaufmann, W., H. Hagemeister, and G. Dirksen. 1980. Adaptation to changes in dietary composition, level and frequency of feeding. In: Y. Ruckebusch, P. Thivend and C. Westport (eds.) Digestive Physiology and Metabolism in Ruminants. p 587-602. AVI Publishing.

Khalil, M. A. K. 2000. Atmospheric methane: An introduction. Atmospheric Methane: Its Role in the Global Environment: 1-8.

Koenig, K.M., Beauchemin, K.A., Rode, L.M., 2003. Effect of grain processing and silage on microbial protein synthesis and nutrient digestibility in beef cattle fed barley-based diets. J. Anim. Sci. 81, 1057-1067.

Kopecny, J., and R. J. Wallace. 1982. Cellular location and some properties of proteolytic enzymes of rumen bacteria. Applied and Environmental Microbiology 43: 1026-1033.

Kotarski, S. F., R. D. Waniska, and K. K. Thurn. 1992. Starch hydrolysis by the ruminal microflora. Journal of Nutrition 122: 178-190.

Lambert, R. J. W., P. N. Skandamis, P. J. Coote, and G. J. E. Nychas. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91: 453-462.

Larraín, R. E., D. M. Schaefer, M. P. Richards, and J. D. Reed. 2008. Finishing steers with diets based on corn, high-tannin sorghum or a mix of both: Color and lipid oxidation in beef. Meat Science 79: 656-665.

Lean, I. J., L. K. Wade, M. A. Curtis, and J. Porter. 2000. New approaches to control of ruminal acidosis in dairy cattle. Asian-Australasian Journal of Animal Sciences 13: 266-269.

Lee, C. H., J. D. Reed, and M. P. Richards. 2006. Ability of various polyphenolic classes from cranberry to inhibit lipid oxidation in mechanically separated turkey and cooked ground pork. Journal of Muscle Foods 17: 248-266.

Lowry, J. B., and P. M. Kennedy. 1996. Fermentation of flavonols by rumen organisms. Proc Aust Soc Anim Prod 21.

Mackie, R. I., C. S. McSweeney, and A. V. Klieve. 2002. Microbial ecology of the ovine rumen. In: M. Freer and H. Dove (eds.) Sheep nutrition. p 71-95. CABI.

Mackie, R. I., B. A. White, and R. E. Isaacson. 1997. Gastrointestinal Microbiology. Chapman & Hall, New York.

Manach, C., A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez. 2004. Polyphenols: Food sources and bioavailability. American Journal of Clinical Nutrition 79: 727-747.

Marino, M., C. Bersani, and G. Comi. 1999. Antimicrobial activity of the essential oils of Thymus vulgaris L. measured using a bioimpedometric method. Journal of Food Protection 62: 1017-1023.

MARM. 2010. Guía de mejores técnicas disponibles del sector porcino. Ministerio de Medio Ambiente, Rural y Marino, Madrid.

Martin, A. K. 1982. The origin of urinary aromatic compounds excreted by ruminants. 2. The metabolism of phenolic cinnamic acids to benzoic acid. British Journal of Nutrition 47: 155-164.

Martin, C., D. P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: From microbe to the farm scale. Animal 4: 351-365.

Martín-Orúe, S.M., Balcells, J., Zakraoui, F., Castrillo, C., 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: Effect of detachment procedure. Anim. Feed Sci.Tech. 71, 269-282.

McAllister, T. A., and K. J. Cheng. 1996. Microbial strategies in the ruminal digestion of cereal grains. Animal Feed Science and Technology 62: 29-36.

McAllister, T. A., K. J. Cheng, L. M. Rode, and C. W. Forsberg. 1990. Digestion of barley, maize, and wheat by selected species of ruminal bacteria. Applied and Environmental Microbiology 56: 3146-3153.

McAllister, T.A., Dong, Y., Yanke, L.J., Bae, H.D., Cheng, K.J., Costerton, J.W., 1993. Cereal grain digestion by selected strains of ruminal fungi. Canadian Journal of Microbiology 39, 367-376.

McAllister, T. A., and C. J. Newbold. 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agr. 48: 7-13.

McSweeney, C. S., and R. I. Mackie. 1996. Gastrointestinal detoxification and digestive disorders in ruminant animals. In: R. I. Mackie and B. A. White (eds.) Gastrointestinal Microbes and Host Interactions. p 583-634. Chapman & Hall, New York.

Mertens, D. R. 1997. Creating a System for Meeting the Fiber Requirements of Dairy Cows. Journal of Dairy Science 80: 1463-1481.

Middleton Jr, E., C. Kandaswami, and T. C. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52: 673-751.

Mirzoeva, O. K., R. N. Grishanin, and P. C. Calder. 1997. Antimicrobial action of propolis and some of its components: The effects on growth, membrane potential and motility of bacteria. Microbiol. Res. 152: 239-246.

Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4: 1024-1036.

Mori, A., C. Nishino, N. Enoki, and S. Tawata. 1987. Antibacterial activity and mode of action of plant flavonoids against Proteus vulgaris and Staphylococcus aureus. Phytochemistry 26: 2231-2234.

Mould, F. L., and E. R. Ørskov. 1983. Manipulation of rumen fluid pH and its influence on cellulolysis in sacco, dry matter degradation and the rumen microflora of sheep offered either hay or concentrate. Animal Feed Science and Technology 10: 1-14.

Mountfort, D. O., and R. A. Asher. 1988. Production of α-Amylase by the Ruminal Anaerobic Fungus Neocallimastix frontalis. Applied and Environmental Microbiology 54: 2293-2299.

Movileanu, L., I. Neagoe, and M. L. Flonta. 2000. Interaction of the antioxidant flavonoid quercetin with planar lipid bilayers. International Journal of Pharmaceutics 205: 135-146.

Mukai, K., S. Mitani, K. Ohara, and S. I. Nagaoka. 2005. Structure-activity relationship of the tocopherol-regeneration reaction by catechins. Free Radical Biology and Medicine 38: 1243-1256.

Mukai, K., K. Sawada, Y. Kohno, and J. Terao. 1993. Kinetic study of the prooxidant effect of tocopherol. Hydrogen abstraction from lipid hydroperoxides by tocopheroxyls in solution. Lipids 28: 747-752.

Murray, R. M., A. M. Bryant, and R. A. Leng. 1976. Rates of production of methane in the rumen and large intestine of sheep. British Journal of Nutrition 36: 1-14.

Nagaraja, T. G., T. B. Avery, E. E. Bartley, S. K. Roof, and A. D. Dayton. 1982. Effect of lasalocid, monensin or thiopeptin on lactic acidosis in cattle. Journal of Animal Science 54: 649-658.

Nagaraja, T. G., T. B. Avery, S. J. Galitzer, and D. L. Harmon. 1985. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. Am. J. Vet. Res. 46: 2444-2452.

Nagaraja, T. G., C. J. Newbold, C. J. Van Nevel, and D. I. Demeyer. 1997. Manipulation of ruminal fermentation. In: P. N. Hobson and C. S. Stewart (eds.) The Rumen Microbial Ecosystem. p 523-632. Chapman and Hall, London, UK.

Nagaraja, T. G., and E. C. Titgemeyer. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. J. Dairy Sci. 90(Suppl. 1): E17-38.

Nakamura, Y., and Y. Tonogai. 2003. Metabolism of Grape Seed Polyphenol in the Rat. Journal of Agricultural and Food Chemistry 51: 7215-7225.

Nijveldt, R. J. et al. 2001. Flavonoids: A review of probable mechanisms of action and potential applications. American Journal of Clinical Nutrition 74: 418-425.

Nocek, J. E. 1997. Bovine Acidosis: Implications on Laminitis. Journal of Dairy Science 80: 1005-1028.

Odom, J. M. 1997. Anthraquinone inhibition of methane production in methanogenic bacteria. Bio-technical, Resources LP. WI, US.

Offner, A., A. Bach, and D. Sauvant. 2003. Quantitative review of in situ starch degradation in the rumen. Animal Feed Science and Technology 106: 81-93.

O'Grady, M. N., F. J. Monahan, and N. P. Brunton. 2001. Oxymyoglobin oxidation and lipid oxidation in bovine muscle - Mechanistic studies. Journal of Food Science 66: 386-392.

Orskov, E. R., W. P. Flatt, and P. W. Moe. 1968. Fermentation balance approach to estimate extent of fermentation and efficiency of volatile fatty acid formation in ruminants. J. Dairy Sci. 51: 1429-1435.

Oskoueian, E., N. Abdullah, and A. Oskoueian. 2013. Effects of Flavonoids on Rumen Fermentation Activity, Methane Production, and Microbial Population. BioMed Res. Int. 2013: 8.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in Cattle: A Review. J. Anim. Sci. 76: 275-286.

Patra, A. K., and J. Saxena. 2009. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. Nutrition Research Reviews 22: 204-219.

Patra, A. K., and J. Saxena. 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71: 1198-1222.

Pazos, M., J. M. Gallardo, J. L. Torres, and I. Medina. 2005. Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. Food Chemistry 92: 547-557.

Pazos, M., S. Lois, J. L. Torres, and I. Medina. 2006. Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. Journal of Agricultural and Food Chemistry 54: 4417-4423.

Pfalzgraf, A., M. Frigg, and H. Steinhart. 1995. α-Tocopherol contents and lipid oxidation in pork muscle and adipose tissue during storage. Journal of Agricultural and Food Chemistry 43: 1339-1342.

Pietta, P. G. 2000. Flavonoids as antioxidants. Journal of Natural Products 63: 1035-1042.

Prior, R. L., and G. Cao. 2000. Antioxidant phytochemicals in fruits and vegetables: Diet and health implications. HortScience 35: 588-592.

Procházková, D., I. Boušová, and N. Wilhelmová. 2011. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82: 513-523.

Ranilla, M. J., D. P. Morgavi, and J. P. Jouany. 2003. Effect of time after defaunation on methane production in vitro. Reproduction, Nutrition, Development 44: 60.

Rasmussen, M. A., R. B. Hespell, B. A. White, and R. J. Bothast. 1988. Inhibitory effects of methylcellulose on cellulose degradation by Ruminococcus flavefaciens. Appl. Environ. Microbiol. 54: 890-897.

Reinli, K., and G. Block. 1996. Phytoestrogen content of foods - A compendium of literature values. Nutrition and Cancer 26: 123-148.

Renerre, M., and R. Labas. 1987. Biochemical factors influencing metmyoglobin formation in beef muscles. Meat Science 19: 151-165.

Rhodes, M. J. C. 1996. Physiologically-active compounds in plant foods: An overview. P. Nutr. Soc. 55: 371-384.

Rice-Evans, C. A., N. J. Miller, P. G. Bolwell, P. M. Bramley, and J. B. Pridham. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radical Research 22: 375-383.

Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20: 933-956.

Rochfort, S., A. J. Parker, and F. R. Dunshea. 2008. Plant bioactives for ruminant health and productivity. Phytochemistry 69: 299-322.

Rouseff, R. L., S. F. Martin, and C. O. Youtsey. 1987. Quantitative survey of narirutin, naringin, hesperidin, and neohesperidin in Citrus. Journal of Agricultural and Food Chemistry 35: 1027-1030.

Russell, J. R., and T. Hino. 1985. Regulation of lactate production in Streptococcus bovis: A spiraling effect that contributes to rumen acidosis. J. Dairy Sci. 68: 1712-1721.

Russell, J. B., and H. J. Strobel. 1989. Effect of ionophores on ruminal fermentation. Applied and Environmental Microbiology 55: 1-6.

Saija, A., Scalese, M., Lanza, M., Marzullo, D., Bonina, F., Castelli, F., 1995. Flavonoids as antioxidant agents: Importance of their interaction with biomembranes. Free Radical Biology and Medicine 19, 481-486.

Santos-Buelga, C., and A. Scalbert. 2000. Proanthocyanidins and tannin-like compounds - Nature, occurrence, dietary intake and effects on nutrition and health. Journal of the Science of Food and Agriculture 80: 1094-1117.

Satter, L. D., and L. L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. British Journal of Nutrition 32: 199-208.

Sauvant, D., F. Meschy, and D. Mertens. 1999. Les composantes de l'acidose ruminale et les effets acidogènes des rations. INRA Prod. Anim. 12: 49-60.

Schelling, G.T., 1984. Monensin mode of action in the rumen. J. Anim. Sci. 58, 1518-1527.

Schneider, H., A. Schwiertz, M. D. Collins, and M. Blaut. 1999. Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. Archives of Microbiology 171: 81-91.

Schoefer, L., Mohan, R., Braune, A., Birringer, M., Blaut, M., 2002. Anaerobic C-ring cleavage of genistein and daidzein by Eubacterium ramulus. FEMS Microbiol. Lett. 208, 197-202.

Schwartzkopf-Genswein, K.S., Beauchemin, K.A., McAllister, T.A., Gibb, D.J., Streeter, M., Kennedy, A.D., 2004. Effect of feed delivery fluctuations and feeding time on ruminal acidosis, growth performance, and feeding behavior of feedlot cattle. Journal of Animal Science 82, 3357-3365.

Selinger, L. B., C. W. Forsberg, and K. J. Cheng. 1996. The rumen: A unique source of enzymes for enhancing livestock production. Anaerobe 2: 263-284.

Sevanian, A., and P. Hochstein. 1985. Mechanisms and consequences of lipid peroxidation in biological systems. Annual Review of Nutrition 5: 365-390.

Silberberg, M., Gil-Izquierdo, A., Combaret, L., Remesy, C., Scalbert, A., Morand, C., 2006. Flavanone metabolism in healthy and tumor-bearing rats. Biomedicine and Pharmacotherapy 60, 529-535.

Silva, M.M., Santos, M.R., Caroço, G., Rocha, R., Justino, G., Mira, L., 2002. Structure-antioxidant activity relationships of flavonoids: A re-examination. Free Radical Research 36, 1219-1227.

Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L., 2012. Summary for Policymakers, in: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.

Spencer, J. P. E., M. M. Abd El Mohsen, A. M. Minihane, and J. C. Mathers. 2008. Biomarkers of the intake of dietary polyphenols: Strengths, limitations and application in nutrition research. British Journal of Nutrition 99: 12-22.

Spencer, J.P.E., Chowrimootoo, G., Choudhury, R., Debnam, E.S., Srai, S.K., Rice-Evans, C., 1999. The small intestine can both absorb and glucuronidate luminal flavonoids. FEBS Letters 458, 224-230.

Stack, R. J., R. E. Hungate, and W. P. Opsahl. 1983. Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8. Appl. Environ. Microb. 46: 539-544.

Steinfeld, H., Gerber, P., Wassenaar, T.D., Castel, V., Rosales, M.M., de Haan, C., 2006. Livestock's long shadow: Environmental issues and options, Food and Agriculture Organization of the United Nations, Roma.

Stumm, C. K., H. J. Gijzen, and G. D. Vogels. 1982. Association of methanogenic bacteria with ovine rumen ciliates. British Journal of Nutrition 47: 95-99.

Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y., 2001. Diet-Dependent Shifts in the Bacterial Population of the Rumen Revealed with Real-Time PCR. Appl. Environ. Microb. 67, 2766-2774.

Tamminga, S. 1979. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. 49: 1615-1630.

Tang, S. Z., J. P. Kerry, D. Sheehan, D. J. Buckley, and P. A. Morrissey. 2000. Dietary tea catechins and iron-induced lipid oxidation in chicken meat, liver and heart. Meat Science 56: 285-290.

Teather, R. M., M. A. Hefford, and R. J. Forster. 1997. Genetics of rumen bacteria. In: P. N. Hobson, Stewart, C.S. (ed.) The Rumen Microbial Ecosystem. p 427-466. Blackie Academic and Professional, London.

Toda, M., S. Okubo, Y. Hara, and T. Shimamura. 1991. Antibacterial and bactericidal activities of tea extracts and catechins against methicillin resistant Staphylococcus aureus. Japanese Journal of Bacteriology 46: 839-845.

Tothi, R., P. Lund, M. R. Weisbjerg, and T. Hvelplund. 2003. Effect of expander processing on fractional rate of maize and barley starch degradation in the rumen of dairy cows estimated using rumen evacuation and in situ techniques. Animal Feed Science and Technology 104: 71-94.

Trenkle, A. 2002. Formulating beef cattle rations for fiber and starch to optimize animal health and productivitiy Intermountain Nutrition Conference Proceedings, Utah State University. Logan, UT.

Trinci, A.P.J., Davies, D.R., Gull, K., Lawrence, M.I., Nielsen, B.B., Rickers, A., Theodorou, M.K., 1994. Anaerobic fungi in herbivorous animals. Mycological Research 98, 129-152.

Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T., Iinuma, M., 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant Staphylococcus aureus. Journal of Ethnopharmacology 50, 27-34.

Tzounis, X., Vulevic, J., Kuhnle, G.G.C., George, T., Leonczak, J., Gibson, G.R., Kwik-Uribe, C., Spencer, J.P.E., 2008. Flavanol monomer-induced changes to the human faecal microflora. Brit. J. Nutr. 99, 782-792.

Van Dijk, C., A. J. M. Driessen, and K. Recourt. 2000. The uncoupling efficiency and affinity of flavonoids for vesicles. Biochemical Pharmacology 60: 1593-1600.

Van Kessel, J. A. S., and J. B. Russell. 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiology Ecology 20: 205-210.

Van Nevel, C. J., and D. I. Demeyer. 1988. Manipulation of rumen fermentation. In: P. N. Hobson (ed.) The Rumen Microbial Ecosystem. p 387-443. Elsevier, London.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. Cornell University Press.

Vaughan, T. A., J. M. Ryan, and N. J. Czaplewski. 2011. Mammalogy. 5th ed. ed. Jones & Bartlett Publishers, Sudbury, MA.

Viuda-Martos, M., Y. Ruiz-Navajas, J. Fernández-López, and J. A. Pérez-Álvarez. 2010. Effect of added citrus fibre and spice essential oils on quality characteristics and shelf-life of mortadella. Meat Science 85: 568-576.

Wallace, R. J. 1994. Ruminal microbiology, biotechnology, and ruminant nutrition: progress and problems. Journal of animal science 72: 2992-3003.

Wallace, R. J. 1996. The proteolytic systems of ruminal microorganisms. Animal Research 45: 301-308.

Wallace, R. J., R. Onodera, and M. A. Cotta. 1997. Metabolism of nitrogen-containing compounds. In: P. N. Hobson and C. S. Stewart (eds.) The Rumen Microbial Ecosystem. p 283-328. Chapman & Hall, London, UK.

Walle, T. 2004. Absorption and metabolism of flavonoids. Free Radical Biology and Medicine 36: 829-837.

Wang, D., Huang, J., Zhang, Z., Tian, X., Huang, H., Yu, Y., Zhang, G., Ding, J., Huang, R., 2013. Influences of Portulaca oleracea extracts on in vitro methane emissions and rumen fermentation of forage. J. Food Agric. Environ. 11, 483-488.

Wang, Y., T. A. McAllister, L. J. Yanke, and P. R. Cheeke. 2000. Effect of steroidal saponin from Yucca schidigera extract on ruminal microbes. Journal of Applied Microbiology 88: 887-896.

Whitelaw, F. G., J. M. Eadie, L. A. Bruce, and W. J. Shand. 1984. Methane formation in faunated and ciliate-free cattle and its relationship with rumen volatile fatty acid proportions. British Journal of Nutrition 52: 261-275.

Williams, A. G., and S. E. Withers. 1991. Effect of ciliate protozoa on the activity of polysaccharide-degrading enzymes and fibre breakdown in the rumen ecosystem. Journal of Applied Bacteriology 70: 144-155.

Winter, J., L. H. Moore, V. R. Dowell Jr, and V. D. Bokkenheuser. 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microb. 55: 1203-1208.

Wright, A.D.G., Kennedy, P., O'Neill, C.J., Toovey, A.F., Popovski, S., Rea, S.M., Pimm, C.L., Klein, L., 2004. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine 22, 3976-3985.

Wu, V. C. H., X. Qiu, B. G. de los Reyes, C. S. Lin, and Y. Pan. 2009. Application of cranberry concentrate (Vaccinium macrocarpon) to control Escherichia coli O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa. Food Microbiol. 26: 32-38.

Yaghoubi, S. M. J., G. R. Ghorbani, H. R. Rahmani, and A. Nikkhah. 2007. In vitro manipulation of rumen fermentation by propolis flavonoids and monensin. J. Dairy Sci. 90(suppl. 1): 105-106.

Yáñez-Ruiz, D. R., B. MacÍas, E. Pinloche, and C. J. Newbold. 2010. The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. FEMS Microbiology Ecology 72: 272-278.

Yang, W. Z., K. A. Beauchemin, K. M. Koenig, and L. M. Rode. 1997. Comparison of Hull-less Barley, Barley, or Corn for Lactating Cows: Effects on Extent of Digestion and Milk Production. Journal of Dairy Science 80: 2475-2486.

Young, J. F., J. Stagsted, S. K. Jensen, A. H. Karlsson, and P. Henckel. 2003. Ascorbic acid, α-tocopherol, and oregano supplements reduce stress-induced deterioration of chicken meat quality. Poultry Science 82: 1343-1351.

Zhu, Q. Y., Y. Huang, D. Tsang, and Z. Y. Chen. 1999. Regeneration of α-tocopherol in human low-density lipoprotein by green tea catechin. Journal of Agricultural and Food Chemistry 47: 2020-2025.

Zinn, R.A., Barreras, A., Owens, F.N., Plascencia, A., 2008. Performance by feedlot steers and heifers: Daily gain, mature body weight, dry matter intake, and dietary energetics. J. Anim. Sci. 86, 2680-2689.

Annex

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

J. Balcells, A. Aris, A. Serrano, A. R. Seradj, J. Crespo and M. Devant

J ANIM SCI 2012, 90:4975-4984.
doi: 10.2527/jas.2011-4955 originally published online July 24, 2012

Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets[1]

J. Balcells,*[2] A. Aris,† A. Serrano,† A. R. Seradj,* J. Crespo,‡ and M. Devant†

*Departament Producció Animal, ETSEA, Alcalde Rovira Roure 191, 25198 Lleida, Spain; †IRTA (Institut de Recerca i Tecnologia Agroalimentàries), and Animal Nutrition, Management, and Welfare Research Group, Torre Marimon, 08140 Caldes de Montbui, Barcelona, Spain; and ‡Exquim S.A., Diagonal 549, 08029 Barcelona, Spain

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

 J. Anim. Sci. 2012.90:4975–4984
doi:10.2527/jas2011-4955

INTRODUCTION

High-concentrate diets can cause rumen fermentation dysfunctions such as rumen acidosis or bloat (Beauchemin and Buchanan-Smith, 1990). The inclusion of antibiotics (e.g., monensin) in the diet appears to reduce the incidence of those rumen dysfunctions; however, antibiotics as feed additives were banned by the European Community (European Communities, 2003) and flavonoids have been proposed as alternatives to antibiotic therapies (Rhodes, 1996; Broudiscou and Lassalas, 2000). Flavonoids are benzo-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

[1]This research was financed by Exquim S.A, San Cugat, Barcelona, and the TRACER project (Ref: PET2008-0116). The authors thank the staff of the IRTA (Experimental station, Prat de Llobregat, Barcelona) and the Chemistry Departments of the IRTA and UAB for their assistance.

[2]Corresponding author: balcells@prodan.udl.cat

Received November 25, 2011.
Accepted June 28, 2012.

anti-inflammatory, antioxidant, and antimicrobial properties (Harborne and Williams, 2000). The effects of flavonoids on rumen fermentation have been the subject of in vitro (Broudiscou and Lassalas, 2000; Yaghoubi et al., 2007) and in vivo experiments (De Freitas et al., 2007). When mixtures of plants flavonoids were tested in a continuous rumen culture system, the flavonoids modified fermentation conditions (pH, propionate proportion, and protein degradation) although the results were not homogeneous (Broudiscou et al., 1999; Broudiscou and Lassalas, 2000).

Bioflavex (**FL**; Exquim S.A., Barcelona, Spain) is a blend of natural flavonoid extracts comprising mostly naringine (200 g/kg), which is extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradisi*; 400 g/kg). From the existing literature it was hypothesized that Bioflavex could exert some antimicrobial activities and the objective of this study was to verify the Bioflavex effects on ruminal pH and specific ruminal bacteria involved in lactate production under 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 experimental station (Prat de Llobregat, Spain). Animals were fed grass hay (11.2% CP, 62.2% NDF, and 34.4% ADF on a DM basis) at a maintenance level (7.2 kg DM/d). The experiment was conducted using a crossover (2 × 2) design that had 2 treatments and 2 periods (22 d each). On d 21 of the experiment, the animals were fasted and on d 22, at 0800 h, rumen acidosis was induced by applying, manually, 5 kg of ground wheat (11.2% CP, 11.0% NDF, and 3.7% ADF on a DM basis) through the cannula. Half of the animals received FL (300 mg/kg), which was mixed in with the ground wheat, and the other half did not receive FL (Control: **CTR**). After a 15-d transition period, the same 22-d experiment was repeated.

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

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

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

Measurements and Sample Collection

Experiment 1. From d 19 to 22 of each experimental period, rumen pH was measured every 22 min using a pH meter (X-Mate Pro MX 300, Mettler-Toledo, Barcelona, Spain) that was capable of recording and storing pH values automatically (for details, see Bach et al., 2007). Samples of rumen contents were collected before (0 h) and 2, 4, 8, and 24 h after heifers were given the ground wheat supplement, which were stored frozen (–20° C) until they were analyzed for lactate, 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 mercuric chloride, 20 mL/L orthophosphoric acid, and 2 g/L 4-methylvaleric acid (internal standard) in distilled water and frozen until the VFA analysis. For the microbial DNA analyses, 50-mL rumen samples were centrifuged at 6500 × *g* for 15 min at 4 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 weighing the animals at 0800 h, the rumen contents (about 200 mL) from the cannulated animals were collected using a vacuum pump, pH was recorded, and the rumen samples were filtered through 2 layers of cheesecloth. Two subsamples were used to quantify NH_3–N and VFA concen-

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_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.

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

[4]Assayed using heat-stable amylase.

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

Chemical and Microbial Analyses

After DM determination using an oven at 60° 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 Analytical Chemists (AOAC, 1990). Dietary NDF and ADF concentrations were measured following the method of Van Soest et al. (1991) using sodium sulfite and α-amylase. To measure rumen 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). Rumen VFA concentrations were measured based on Jouany (1982) method. L+ plus D-lactate was determined by the colorimetric method proposed by Taylor (1996). Urinary concentrations of allantoin, uric acid, and creatinine were measured using the HPLC method described by Balcells et al. (1992). Total microbial DNA was extracted using the repeated bead beating and column (**RBB+C**) method (Yu and Morrison, 2004) using bead beating in the presence of high concentrations of SDS, salt, and EDTA, and subsequent DNA purifications were performed using QIAamp columns from the Stool DNA Kit (QIAGEN, Valencia, CA).

Quantitative Real-Time PCR (**RT-PCR**) was performed using 0.2-mL 96-well plates, IQSYBR Green Supermix, and the MyIQ Real-Time Detection System from BioRad (Hercules, CA). For the bacteria quantification, specific primers for regions of the 16S rRNA gene were used at 0.5 μM final concentration. The PCR amplification cycles and primer sequences are presented in Table 2. Amplicon specificity was assessed using melting curve analyses of the PCR end products by increasing the temperature from 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 plasmids derived from pGEM-T vectors (Invitrogen, Carlsbad, CA), which carried the specific amplicons from *Selenomonas ruminantium* and *Streptococcus bovis* as standards.

Calculations and Statistical Analyses

Urinary PD:creatinine ratios were measured in the spot samples that were collected from the cannulated heifers. Absolute values of PD (mmol/d) were calculated assum-

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

Microorganism	*16SrRNA* Primers	Reference	RT-PCR amplification cycle
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)

ing that creatinine excretion depends on body mass only (Van Niekerk et al., 1963) and then creatinine excretion rate should be equivalent to 896 mmol/kg $BW^{0.75}$ reported by 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 effect. All of the animals were weighed at the beginning of the experiment and thereafter once a week until the end of the experiment, and the data were used to calculate the ADG as the slope of the linear regression of BW against time. For the rumen and urine data, the effect of time after feeding (hours) and interactions were considered as fixed effects. In the model, pen (n = 8) was the experimental unit for all of the statistical analyses. Significant differences and tendencies were declared at $P < 0.05$ and $P < 0.10$, respectively.

RESULTS

Experiment 1

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

Changes in the rumen pH during the 72-h period are shown in Figure 1. The interaction between treatment and feeding phase (i.e., maintenance, fasting, and acidosis induction) on rumen pH was significant ($P < 0.01$). After the ground wheat was administered, rumen pH decreased, more so in the CTR (5.98; SEM = 0.029) than in the FL heifers (6.29; SEM = 0.031). Mean values of ruminal NH_3–N and VFA concentrations and relative VFA proportions are presented in Table 3. Treatment did not affect total VFA concentration or molar proportions of propionate in the rumen. As expected, and paralleling the changes in rumen pH, total rumen VFA concentrations and molar proportions of propionate increased at 2 and 4 h after ground wheat was administered through a rumen cannula; however, at 8 h, concentrations declined. Compared with the CTR heifers, the FL heifers had a decreased rumen molar proportion of acetate ($P < 0.05$) and tended to have reduced concentrations of butyrate ($P = 0.06$) and acetate to propionate ratio ($P = 0.09$). The posttreatment changes in rumen acetate and butyrate (mol/100 mol) were similar to those that were observed in VFA concentrations.

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

Experiment 2

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

Average pH (6.42 vs. 6.09; SEM = 0.03) and VFA concentrations (74.8 vs. 65.7 m*M*; SEM = 1.86) in cannulated 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 administration. Ammonia-N concentrations were less in the FL than in the CTR heifers [10.2 vs. 41.5 mg/L (SEM = 2.81), respectively].

Rumen molar proportions of acetate (53.9 vs. 59.4 mol/100 mol; SEM = 0.60) were less ($P < 0.01$) and proportions of propionate (35.5 vs. 28.1 mol/100 mol; SEM = 0.57) were greater ($P < 0.01$) in the FL than in the CTR heifers; consequently, the acetate-to-propionate ratio was greater ($P < 0.01$) in the CTR heifers than in

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.

the FL heifers [2.34 vs. 1.65 (SEM = 0.06), respectively]. 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 performance can be diminished (Owens et al., 1998). Chronic and acute acidosis are indicated by rumen pH values of 5.6 and 5.2, respectively (Cooper and Klopfenstein, 1996). In Exp. 1, in which a wheat supplement was used to induce acidosis, rumen pH was reduced from 6.70 [SEM = 0.22; which is within the normal range for roughage diets (France and Siddons, 1993)] to 5.22 (SEM = 0.19), and the average amount of time in which pH was <5.5 and <6.0 was 0.5 and 4 h, respectively. The minimum pH values (5.0 to 5.5) registered were within the pH ranges reported in studies that used grain engorgement to induce subacute acidosis in (Bauer et al., 1995; Krehbiel et al., 1995; Goad et al., 1998).

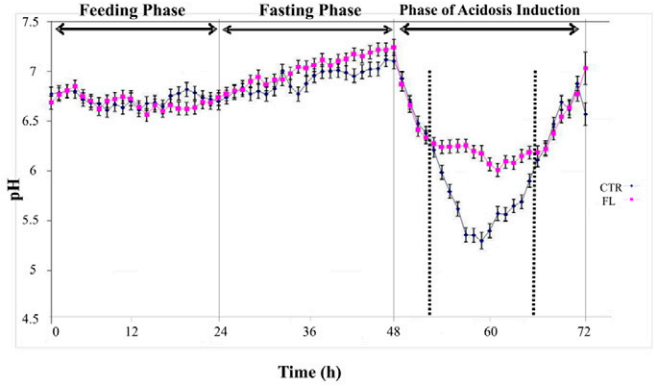


Figure 1. Rumen pH over time (hours) where Holstein crossbreed heifers were fed, i) at a maintenance level (feeding phase), ii) 1 d before acidosis was induced (fasting phase, 24 h), and, iii) onset of acidosis induction. Animals were supplied with Bioflavex (FL) or used as a control (CTR), and the dotted 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.

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

Table 4. Effects of Bioflavex before (0 h) and after onset (4 + 8 h) of induced acidosis on lactate concentrations (mmol/L) and rumen populations of *Selenomonas ruminantium*, *Streptococcus bovis*, and *Megasphaera elsdenii* (Exp.1)

	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	0.97	0.80	0.08	0.41	1.35	0.24	0.44	<0.01	0.09
Absolute quantification[4]									
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).

daidzein has been demonstrated to be able to modify the predominant microbiota (Schoefer et al., 2002; Clavel et al., 2005). Naringin, the main component of Bioflavex, degrades to aglycone (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 antimicrobial 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 rumen ecosystem adapted to the presence of polyphenol compounds.

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

[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]Tr = treatment effect; W = week effect; Tr × W = treatment by week effect.

[3]Time corresponds to BW and feed intake of animal, which were recorded weekly.

[4]Feed efficiency = feed intake:ADG (kg/kg).

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

Table 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 = 8)	FL (n = 8)	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	1.30	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).

(or both) on rumen pH; however, the numerical less lactate concentration in CTR than in FL heifers at 8 h after wheat supply may indicate that FL supplementation prevented heifers from lactic acidosis. Nagaraja and Titgemeyer (2007) indicated that in subacute acidosis, the reason for pH to decline below 5.6 is accumulation of VFA, which is a combination of overproduction (increased substrate) and possibly decreased absorption. Although lactic acid is produced during subacute acidosis, it does not accumulate because lactate-fermenting bacteria remain active and rapidly metabolize it to VFA However, as the pH nears 5.0 or below for a sustained period, the growth of lactate-fermenting bacteria is inhibited, and hence lactate begins to accumulate. Therefore, subacute acidosis has

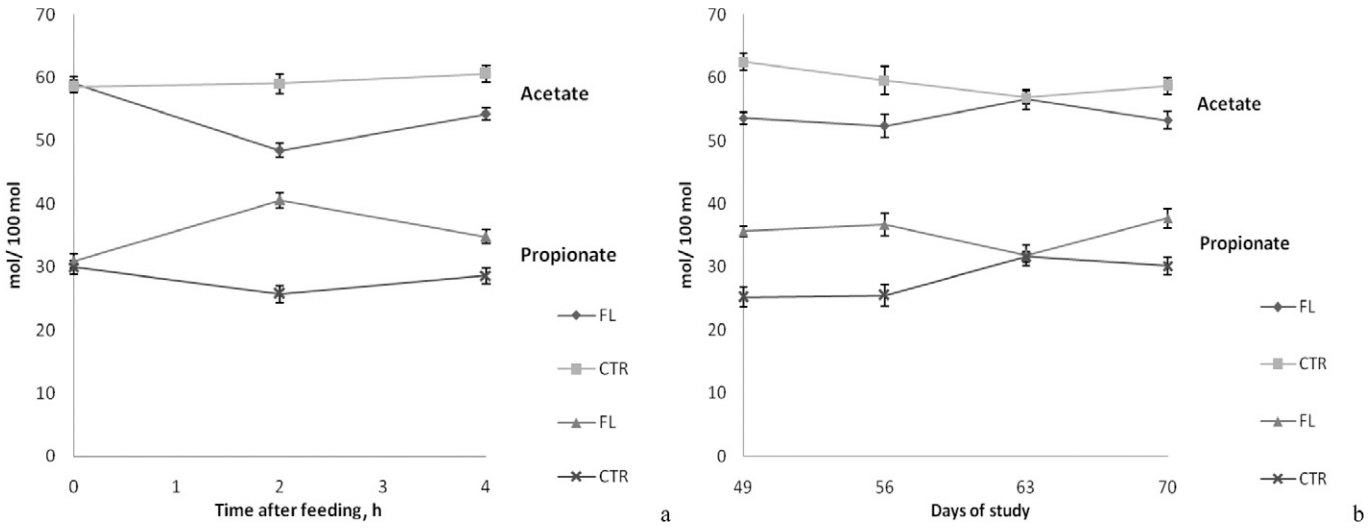


Figure 2. Changes in VFA concentration registered in response to a commercial flavonoid supplement (Bioflavex; Exquim S.A., Barcelona, Spain) in 16 rumen-cannulated Fleckvieh heifers (Exp. 2) in relation to time after concentrate was administered [a) interaction of treatment × time after concentrate was given] and over the course of the experiment [b) interaction of treatment × days of experiment (sampling) for acetate and propionate (mol/100 mol)]. CTR = control (did not receive Bioflavex); FL = heifers supplemented with Bioflavex.

the potential to become lactic acidosis if the pH of 5.0 is sustained for a time (Nagaraja and Titgemeyer, 2007). In Exp. 2, heifers supplemented FL consumed more straw than CTR heifers; this may stimulate rumination and saliva production buffering rumen pH, explaining the greater pH observed in FL compared with CTR heifers even if rumen VFA concentration was greater in FL than in CTR heifers. As mentioned previously, correlation between rumen VFA concentration and pH is low, and buffering mechanisms such as saliva have a great impact on rumen pH regulation. Mechanisms whereby flavonoid supplementation may stimulate straw consumption are unknown. So, flavonoids addition seems to be effective in preventing pH reduction through modifying the activity of lactating-consuming bacteria but also may have a direct buffer effect or stimulating straw consumption or both. In both of our experiments, flavonoid supplements increased rumen molar proportions of propionate and reduced the acetate-to-propionate ratio, which suggests that flavonoid supplements might have altered the entire rumen microbiota and stimulated the growth of propionate-producing bacteria. Furthermore, FL supplements reduced rumen NH_3–N concentrations and increased urinary PD excretion (an index of the duodenal flow of microbial N). The reduction in rumen ammonia concentrations coupled with a significant increase in the duodenal flow of microbial N suggests an improvement in rumen N use.

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

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

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

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

In summary, flavonoid supplementation might be effective in improving rumen fermentation and reducing the incidence of rumen acidosis. This effect of flavonoids may be partially explained by increasing the numbers of lactate-consuming microorganisms (e.g., *M. 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.

LITERATURE CITED

Association of Official Analytical Chemists (AOAC). 1990. Official Methods of Analysis. 15th ed. AOAC, Arlington, VA.

Bach, A., C. Iglesias, and M. Devant. 2007. Daily rumen pH pattern of loose-housed dairy cattle as affected by feeding pattern and live yeast supplementation. Anim. Feed Sci. Tech. 136:146–153.

Balcells, J., J. A. Guada, J. M. Peiro, and D. S. Parker. 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. J. Chromatogr. 575:153–157.

Bauer, M. L., D. W. Herold, R. A. Britton, R. A. Stock, T. J. Klopfenstein, and D. A. Yates. 1995. Efficacy of laidlomycin propionate to reduce ruminal acidosis in cattle. J. Anim. Sci. 73:3445–3454.

Beauchemin, K. A., and J. G. Buchanan-Smith. 1990. Effects of fiber source and method of feeding on chewing activities, digestive function, and productivity of dairy cows. J. Dairy Sci. 73:749–762.

Bevans, D. W., K. A. Beauchemin, K. S. Schwartzkopf-Genswein, J. J. McKinnon, and T. A. McAllister. 2005. Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle. J. Anim. Sci. 83:1116–1132.

Broudiscou, L. P., and B. Lassalas. 2000. Effects of *Lavandula officinalis* and *Equisetum arvense* dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40:431–440.

Broudiscou, L. P., Y. Papon, and A. Broudiscou. 1999. Effects of inorganic nitrogen and amino acids on the degradation of ammonia-treated barley straw and proteosynthesis in a continuous culture of rumen microbes. Anim. Feed Sci. Tech. 77:149–162.

Burrin, D. G., and R. A. Britton. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63:888–893.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130–132.

Clavel, T., G. Henderson, C. A. Alpert, C. Philippe, L. Rigottier-Gois, J. Doreì, and M. Blaut. 2005. Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. Appl. Environ. Microb. 71:6077–6085.

Coe, M. L., T. G. Nagaraja, Y. D. Sun, N. Wallace, E. G. Towne, K. E. Kemp, and J. P. Hutcheson. 1999. Effect of virginiamycin on ruminal fermentation in cattle during adaptation to a high concentrate diet and during an induced acidosis. J. Anim. Sci. 77:2259–2268.

Cooper, R., and T. Klopfenstein. 1996. Effect of rumensin and feed intake variation on ruminal pH. Pages A1–A14 in Scientific Update on Rumensin/tylan/micotil for the Professional Feedlot Consultant. Elanco Animal Health, Indianapolis, IN.

De Freitas, J. A., J. C. De Souza, R. P. Lana, R. P. Antonangelo, A. A. De Freitas, and R. T. S. De Santana. 2007. Ethanolic extract of propolis in lactating cows. J. Dairy Sci. 90(Suppl. 1):105. (Abstr.)

Decroos, K., S. Vanhemmens, S. Cattoir, N. Boon, and W. Verstraete. 2005. Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. Arch. Microbiol. 183:45–55.

Devant, M., A. Anglada, and A. Bach. 2007. Effects of plant extract supplementation on rumen fermentation and metabolism in young Holstein bulls consuming high levels of concentrate. Anim. Feed Sci. Tech. 137:46–57.

Devant, M., A. Ferret, S. Calsamiglia, R. Casals, and J. Gasa. 2001. Effect of nitrogen source in high-concentrate, low-protein beef cattle diets on microbial fermentation studied in vivo and in vitro. J. Anim. Sci. 79:1944–1953.

European Communities. 2003. Regulation EC No 1831/2003 of the European Parliament and Council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur. Union L268:29–43.

France, J., and R. C. Siddons. 1993. Volatile fatty acid production. Pages 196–203 in Quantitative Aspects Ruminant Digestion and Metabolism. J. M. Forbes and J. France, ed. CAB International, Willingford, UK.

Gibson, G. R., E. R. Beatty, X. Wang, and J. H. Cummings. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108:975–982.

Gladine, C., E. Rock, C. Morand, D. Bauchart, and D. Durand. 2007. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Brit. J. Nutr. 98:691–701.

Goad, D. W., C. L. Goad, and T. G. Nagaraja. 1998. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. J. Anim. Sci. 76:234–241.

González, L. A., L. B. Correa, A. Ferret, X. Manteca, J. L. Ruíz-de-la-Torre, and S. Calsamiglia. 2009. Intake, water consumption, ruminal fermentation, and stress response of beef heifers fed after different lengths of delays in the daily feed delivery time. J. Anim. Sci. 87:2709–2718.

Harborne, J. B., and C. A. Williams. 2000. Advances in flavonoid research since 1992. Phytochemistry 55:481–504.

Jouany, J. P. 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments 2:131–144.

Krehbiel, C. R., R. A. Britton, D. L. Harmon, T. J. Wester, and R. A. Stock. 1995. The effects of ruminal acidosis on volatile fatty acid absorption and plasma activities of pancreatic enzymes in lambs. J. Anim. Sci. 73:3111–3121.

Martín-Orúe, S. M., J. Balcells, J. A. Guada, and M. Fondevila. 2000. Microbial nitrogen production in growing heifers: Direct measurement of duodenal flow of purine bases versus urinary excretion of purine derivatives as estimation procedures. Anim. Feed Sci. Tech. 88:171–188.

Martín-Orúe, S. M., J. Balcells, F. Zakraoui, and C. Castrillo. 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: Effect of detachment procedure. Anim. Feed Sci. Tech. 71:269–282.

Middleton, Jr., E., C. Kandaswami, and T. C. Theoharides. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52:673–751.

Nagaraja, T. G., and E. C. Titgemeyer. 2007. Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. J. Dairy Sci. 90(Suppl. 1):E17–38.

Nagaraja, T. G., T. B. Avery, S. J. Galitzer, and D. L. Harmon. 1985. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. Am. J. Vet. Res. 46:2444–2452.

Ouwerkerk, D., A. V. Klieve, and R. J. Forster. 2002. Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92:753–758.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in cattle: A review. J. Anim. Sci. 76:275–286.

Pérez, J. F., J. Balcells, J. A. Guada, and C. Castrillo. 1997. Rumen microbial production estimated either from urinary purine derivative excretion or from direct measurements of 15N and purine bases as microbial markers: Effect of protein source and rumen bacteria isolates. Anim. Sci. 65:225–236.

Rhodes, M. J. C. 1996. Physiologically-active compounds in plant foods: An overview. P. Nutr. Soc. 55:371–384.

Russell, J. R., and T. Hino. 1985. Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that contributes to rumen acidosis. J. Dairy Sci. 68:1712–1721.

Sauvant, D., F. Meschy, and D. Mertens. 1999. Les composantes de l'acidose ruminale et les effets acidogènes des rations. (In French.) INRA Prod. Anim. 12:49–60.

Schelling, G. T. 1984. Monensin mode of action in the rumen. J. Anim. Sci. 58:1518–1527.

Schoefer, L., R. Mohan, A. Braune, M. Birringer, and M. Blaut. 2002. Anaerobic C-ring cleavage of genistein and daidzein by *Eubacterium ramulus*. FEMS Microbiol. Lett. 208:197–202.

Stack, R. J., R. E. Hungate, and W. P. Opsahl. 1983. Phenylacetic acid stimulation of cellulose digestion by *Ruminococcus albus* 8. Appl. Environ. Microb. 46:539–544.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microb. 67:2766–2774.

Taylor, K. A. C. C. 1996. A simple colorimetric assay for muramic acid and lactic acid. Appl. Biochem. Biotechnol. Enzym. Eng. Biotechnol. 56:49–58.

Tzounis, X., J. Vulevic, G. G. C. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. Kwik-Uribe, and J. P. E. Spencer. 2008. Flavanol monomer-induced changes to the human faecal microflora. Brit. J. Nutr. 99:782–792.

Van Niekerk, B. D., J. T. Reid, A. Bensadoun, and O. L. Paladines. 1963. Urinary creatine as an index of body composition. J. Nutr. 79:463–473.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccha-

rides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597.
Winter, J., L. H. Moore, V. R. Dowell, Jr., and V. D. Bokkenheuser. 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microb. 55:1203–1208.
Wu, V. C. H., X. Qiu, B. G. de los Reyes, C. S. Lin, and Y. Pan. 2009. Application of cranberry concentrate (*Vaccinium macrocarpon*) to control *Escherichia coli* O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa. Food Microbiol. 26:32–38.
Yaghoubi, S. M. J., G. R. Ghorbani, H. R. Rahmani, and A. Nikkhah. 2007. In vitro manipulation of rumen fermentation by propolis flavonoids and monensin. J. Dairy Sci. 90(Suppl. 1):105–106. (Abstr.)
Yu, Z., and M. Morrison. 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microb. 70:4800–4806.

Effects of the flavonoid extract Bioflavex or its pure components on rumen fermentation of intensively reared calves

Journal:	*Journal of Animal Science*
Manuscript ID:	E-2014-8138
Manuscript Type:	Animal Nutrition - Ruminant
Date Submitted by the Author:	03-Jun-2014
Complete List of Authors:	Seradj, Ahmad Reza; University of Lleida, Animal Production Gimeno, Antonio; Universidad de Zaragoza, Producción Animal y Ciencia de los Alimentos Fondevila, Manuel; Universidad de Zaragoza, Producción Animal y Ciencia de los Alimentos Crespo, Javier; Interquim S. A. (FerrerHealthTech), Balcells, Joaquim; University of Lleida, Animal Production
Key Words:	flavonoids, holstein calves, intensive beef production, in vivo, in vitro, rumen fermentation

Running head: Enhancing rumen fermentation using flavonoids

Effects of the flavonoid extract Bioflavex or its pure components on rumen fermentation of intensively reared calves[1]

A. R. Seradj†, A. Gimeno‡, M. Fondevila‡, J. Crespo¶ and J. Balcells†[2]

†Departament Producció Animal, ETSEA, Alcalde Rovira Roure 191, 25198 Lleida, Spain.

‡Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain.

¶Interquim S. A. (FerrerHealthTech), Sant Cugat, Barcelona, Spain.

[1] This research was financed by Interquim S.A. (FerrerHealthTech), Sant Cugat, Barcelona

[2]Corresponding author: J. Balcells; Tel: +34 97 370 64 98

Email: balcells@prodan.udl.cat

ABSTRACT

Two experiments were performed to study the effects of the citrus flavonoids extract Bioflavex (Interquim S.A., FerrerHealthTech, Sant Cugat, Barcelona, Spain; **BF**) on the fermentation characteristics of high concentrate diets. In an *in vivo* experiment, 8 Friesian calves (398 ± 12.2 kg body weight, BW) fitted with a rumen canula were given a basal concentrate (**CTR**) or CTR supplemented with BF (450 mg/kg dry matter, DM) in a 2×4 cross-over design. No differences were observed in concentrate and straw intake, average daily gain (ADG) or feed conversion ratio (FCR) between BF and CTR calves after 24 d of experiment. When rumen contents were sampled at 0, 4 and 8 h post feeding, it was observed that BF improved pH values (6.1 vs. 5.8, $P < 0.01$) and molar proportion of propionate (24.2 vs. 22.5 %, $P < 0.01$), whereas BF reduced lactate concentration (2.08 vs. 0.91 mmol/L, $P < 0.01$) and improved the relative abundance of lactate-consuming microorganism such as *Selenomomas ruminantium* ($P < 0.01$) and *Megaesphaera elsdenii* ($P = 0.06$), although did not alter that of the lactate-producing *Streptococcus bovis*. In an *in vitro* experiment, the effect of BF and its pure flavonoid components added to the mixture was studied, with inoculum from 4 steers given a high concentrate diet,in four experimental series. Bioflavex and its main components such as naringine (**NG**), neohesperidine (**NH**) and poncirine (**PC**) were added to the incubation medium at 500 µg/g DM, with the unsupplemented substrate also included as a control (CTR). Up to 8 h post incubation, BF and its main components (NH and PC) reduced ($P < 0.01$) the volume of gas produced and the molar proportion of acetate ($P < 0.01$), increasing that of propionate ($P < 0.01$). PC reduced the relative quantification of *S. bovis,* whereas NH and BF increased the relative quantification of *M. elsdenii* in relation to CTR ($P < 0.01$). BF supplementation in calves receiving a high concentrate diet was effective in preventing collapses in pH and enhanced rumen fermentation efficiency through modifying the activity of lactating-consuming bacteria and

modulating rumen fermentation towards a greater molar proportion of propionate and a reduction of that of acetate, which may suggest that flavonoid supplementation might have a role in modulating the activity of rumen microbiota.

Key words: flavonoids, holstein calves, intensive beef production, *in vivo*, *in vitro*, rumen fermentation

INTRODUCTION

Manipulation of rumen fermentation to improve efficiency is the main aim of using feed additives in ruminant animals. Promoting the improvement of volatile fatty acids (VFA) together with a reduction of ammonia rumen concentration and methane production are considered as desirable changes in rumen environment (Bodas et al., 2012). Antibiotic therapies have been used to alleviate rumen fermentation dysfunctions (i.e., acidosis or bloat) when high concentrate rations are given to improve rumen efficiency and animal performances (Beauchemin and Buchanan-Smith, 1990). However, massive environmental widespread of antibiotic associated with potential cross-over resistance to human therapies, together with consumers demands on food quality and safety promoted the ban of dietary antibiotic administration in Europe (European Communities, 2003). Removal of antibiotics conventionally used as feed additives forced nutritionists to explore some natural less aggressive substances as alternatives (Patra and Saxena, 2009). Secondary plant extracts (e.g. flavonoids) have been proposed as an alternative to antibiotic therapies (Rhodes, 1996). Balcells et al. (2012) previously showed that addition of Bioflavex (BF; Interquim S.A., Barcelona, Spain), a blend of natural flavonoids extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*), was effective in alleviating reductions in rumen pH in heifers subjected to experimentally induced acidosis. It also improves rumen molar proportions of propionate and prevents lactate accumulation by creating conditions that favored lactate-consuming microorganisms such as *Megasphaera elsdenii*. The objectives of

the present work were, i) to confirm *in vivo* the effects of Bioflavex on rumen metabolism using rumen fistulated calves fed high concentrate rations and, ii) to estimate the contribution of each pure flavonoid as component of the commercial mixture in an *in vitro* system, to simulate *in vivo* rumen fermentation.

MATERIALS AND METHODS

In vivo trial

This experiment was carried out in the facilities of the Servicio de Experimentación Animal (SEA) 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 EU Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Eight crossed, 9 month old Holstein-Frisian calves (398 ± 12.2 kg) were fitted with a canula (88 mm length and 10 mm i.d. DIVASA Farmavic S.A, Vic, Barcelona, Spain) in the dorsal sac of the rumen. After recovery from canulation, animals were housed in 3.2 x 1.7 m individual pens with concrete floor, 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 with the commercial mixture of flavonoids Bioflavex comprising mostly Naringine (NG, 200 g/kg), which is extracted from bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradis*) (400 g/kg composed mainly by Naringine **NG**; Neohesperidine; **NH**, , and Poncirine; **PC**, plus tracer amounts of Hesperidine; **HS** Isonaringina; **IN** and Neoeritrocine; **NE**). The experiment lasted for 24 days and was organized in a 2×4 cross over design, with two periods of 12 days and four calves per period and treatment. Concentrate and straw were supplied *ad libitum* in separated feeders, the

former in only one dose (at 0800 h) and the latter in three daily doses, and consumptions and orts were daily recorded on DM basis. Calves were weighed individually when starting and at the end of the trial. The last two days of each experimental period, 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 (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 ° C until further molecular analysis. Other two 4 mL subsamples were pipetted to tubes containing either 1 mL of a solution made up with 20 mL/L ortophosphoric acid and 2 mL/L of 4-methylvaleric acid as internal marker or 4 mL of HCl 0.2 *N*, that were stored at -20 ° C until further analysis of volatile fatty acids (VFA) and ammonia (NH_3) concentration, respectively.

In vitro trial

Four incubations series were conducted to evaluate the effect of the Bioflavex mixture (BF) or its pure flavonoid components (NG; NH, PC, HS, IN and NE) included at 500 µg/g DM of substrate, on *in vitro* rumen fermentation (Theodorou et al., 1994) using rumen liquor from four rumen cannulated growing steers fed a high concentrate diet (90 % of a commercial concentrate and 10 % barley straw; Table 1) given *ad libitum* for at least 4 weeks. For each incubation series, the rumen contents were collected from a different animal, filtered through a double layer of gauze and used immediately as inoculum (10 % of total incubation volume). Serum glass bottles of 120 mL total volume (four bottles per 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. A mixture of 600 mg of the same concentrate given to steers plus 60 mg

barley straw was used as substrate. 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 ° C in a shaking water bath for 12 h.

Pressure measurements were determined with a TP704 Manometer (DELTA OHM, Italy) at 2, 4, 6, 8, 10 and 12 h after the onset of the 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 DM. At the onset (Time 0) of each incubation series, two samples were taken from the 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 were weighed, immediately frozen in liquid N and stored at -80 ° C for microbiota analyses. The remaining content was filtered through a metal sieve (1 mm mesh size) and sampled for subsequent analyses of ammonia nitrogen and VFA concentration, as above. Samples were immediately frozen (-20 º C) until further analyses.

Microbiological and chemical analyses

The DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions. Relative abundance of *Streptococcus bovis*, *Selenomonas ruminantium* and *Megasphaera elsdenii* in relation to the total bacteria were determined using specific primers. The relative quantification was carried out using $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Analyses were performed on CFX96 Touch real-time PCR detection system (BioRad, Laboratories Inc., Hercules, CA, USA). The primer sets and qPCR condition were described in Table 2.

The DM content of the concentrate and straw was determined at 105 ° C until a constant sample weight. Ash content was determined by incineration on muffle furnace (550 ° C for 4

h) and crude protein (CP) was analyzed according to AOAC (1990). The proportion of neutral detergent fiber (NDF) was determined according Van Soest et al. (1991) procedures, using alpha amylase, and discounting ashes from the residue. Ammonia concentration was determined by the Chaney and Marbach (1962) method after sample centrifugation (2500 x g, 20 min). The VFA concentrations were determined by gas chromatography (GC), based on the technique proposed by Jouany (1982) using a capillary column (BP21 30 m x 0.25 mm ID x 0.25 μm, DE, USA). Lactate was measured using the method of Taylor (1996).

Statistical Analysis

In vivo rumen fermentation parameters (pH, ammonia and VFA concentration and proportions of bacterial species) were analyzed as a 2×4 cross over design using the mixed procedure of SAS (SAS, Inst. Inc., Cary, NC). The model included phase, treatment, sampling day and time post-feeding (hours, considered as repeated measures) and their interactions as fixed factors. Animal within period was considered as the experimental unit.

Results from the *in vitro* trial were studied as a completely randomized block design, occurring in 4 different blocks. Each animal donor, which agrees with the incubation series, was considered as the experimental unit. Computations were performed using the mixed procedure of SAS (SAS, Inst. Inc., Cary, NC), each experiment series being considered as a random effect. The model included block, treatment and their interactions as fixed factors.

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

RESULTS

In vivo trial

There were no differences between CTR and BF in daily intake of concentrate (7.42 vs. 7.79 kg DM/d, SEM = 0.28) or straw (0.83 vs. 1.05 kg DM/d, SEM = 0.16), nor in weight gain

(1.4 vs. 1.4 kg/d, SEM = 0.12) along the experimental period. Mean values of pH, ruminal NH_3 and VFA concentrations and relative molar VFA proportions are presented in Table 3 The average pH was higher ($P < 0.01$) in calves given BF than CTR (6.1 vs. 5.8; SEM = 0.05), and it dropped significantly 4 h after feeding ($P < 0.01$). No interaction between both factors was recorded. Average concentration of NH_3 was higher in CTR ($P < 0.01$), and it was reduced after feeding ($P < 0.01$). Total VFA concentration was not affected by the flavonoid supplementation, but it increased from 0 to 4 h after feeding ($P < 0.01$). Despite molar proportion of propionate increased with flavonoid addition ($P = 0.05$), this only occur at 0 h, whereas no treatment differences were recorded at 4 and 8 h (interaction treatment x hour; $P < 0.01$; Fig. 1a). The opposite trend occurred with acetate proportion, that was lower at 0 h with BF but did not differ afterwards (interaction treatment x hour; $P < 0.01$; Fig. 1b). No treatment differences were recorded for molar proportions of the other VFAs, despite that of butyrate increased, and isobutyrate and isovalerate decreased, from 0 to 4 h ($P < 0.01$).

Rumen lactate concentration and relative proportions of the lactate-producing bacteria *S. bovis* and the lactate-consuming bacteria *S. ruminantium* and *M. elsdenii,* are presented in Table 4. 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, but it decreased linearly with time ($P = 0.016$). In contrast, the average abundance of *M. elsdenii* ($P = 0.06$) and *S. ruminantium* ($P < 0.01$) increased with BF. However, whereas concentration of *M. elsdenii* increased with time ($P = 0.02$), that of *S. ruminantium* showed an unequal evolution: no differences among treatments were observed at 0 and 2 hours after feeding, but at 8 hours relative proportion *S. ruminantium* increase in BF supplemented animals whereas decrease in CTR (Interaction treatment x hour; $P < 0.05$)

In vitro trial

In vitro gas production values from the six selected times of incubation are presented in Table 5. Addition of BF to the culture media reduced gas production ($P < 0.01$) in relation to the CTR, as it was also the case for its main components NH and PC ($P < 0.05$). Although NG also reduced numerically gas production, its effect did not reach statistical significance. Tracer components did not alter gas production in relation to values recorded with CTR bottles.

Table 6 shows ammonia and VFA concentrations of the 12 h *in vitro* incubation trial. Addition of flavonoids has a small effect on NH_3 concentration, and only a slight but significant ($P = 0.03$) reduction in bottles supplemented with the tracer component IN was detected. No treatment differences were recorded ($P > 0.10$) on the total VFA concentration, nor on molar proportions of butyrate and the minor VFAs. However, flavonoid supplementation increased ($P < 0.01$) the molar proportion of propionate at the expense of acetate for either BF or each one of its components, in relation to CTR.

The numerical differences observed in lactate concentrations after 12 h incubation *in vitro* in BF and its main components NG and NH compared with CTR did not reach statistical significance (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 leads to a rumen pH reduction and may induce sub-acute (pH 5.6 to 6.0, Goad et al., 1998) or acute (pH below 5.6, Nagaraja et al., 1985) acidosis. Rumen acidity is provoked by a reduction in salivation, due to shortage of fiber size, and an increase in VFA production, including lactic acid accumulation. Moreover, starch availability improves the presence of glucose-utilizing bacteria through the lactate

pathway (such as *S. bovis*) and the higher lactate concentration may consequently increase to a certain extent the titers 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, BF addition was effective in preventing pH reduction (Balcells et al., 2012); however, in a simultaneous trial using growing heifers the positive effect of the citrus flavonoids blend was less conclusive at the rumen metabolism level and no improvement in ADG was detected. This more controlled in vivo assay using cannulated animals may improve the understanding of BF effect on rumen environment, and the *in vitro* simulation trial of rumen fermentation allows for identify the effectiveness of the pure flavonoid components into the BF mixture.

Calves consumed 88 to 90% concentrate in diet, and consequently rumen pH values dropped to sub-acute values from 4 to 8 h after feeding (Goad et al., 1998). Allowing the animals to adapt to the high-grain diets and the low degradability of corn starch might probably reduce the possibility of acute acidosis (Owens et al., 1998). Average recorded pH and its daily evolution in CTR calves agrees well with previous reports under similar experimental conditions (Beauchemin et al., 2001; Koenig et al., 2003). Confirming our previous work (Balcells et al., 2012), BF addition increased average pH on 0.3 pH units, and BF fed calves apparently tended to a faster recovery of normal pH values after concentrate feeding than those given CTR (5.70 vs. 5.34 at 8 h post-feeding, SEM = 0.08; $P < 0.05$).

It is logical to expect that the effect of BF on microbial environment should relay on the activity of the main flavonoid components of the mixture (i.e., NG, NH and PC). Effectively, NG and NH reduced gas production and microbial activity at a similar level. Moreover, the relationship between 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. This fact would

confirm previous results from our group (Seradj et al., 2014b), who cannot demonstrate any positive effect between over-dosage of flavonoids and methane production under similar experimental conditions.

Anti-microbial activity of flavonoids on pathogenic (Wu et al., 2009) and nonpathogenic (Broudiscou and Lassalas, 2000) bacteria have been reported, and a hypothetical mechanism through the cleavage of C-ring in the flavonoids to produce the toxic phenolic acids has been described (Schoefer et al., 2002). The degradation of NG (as the main component of BF) to the corresponding aglycone (Gladine et al., 2007) and further C-ring cleavage to produce anti-microbial compounds (Winter et al., 1989) would explain some anti-microbial effect related to both NG and BF. Although it is true that the improvement in pH values and the significant reduction in gas production may promote a reduction in rumen microbial fermentation, such anti-microbial activity must have some specificity, given that titters of lactate-consuming microorganism were not reduced, but even increased. Authors are not aware of any anti-microbial metabolic pathway for NH but probably the similarity between both flavonoid structures may suggest a similar metabolic pathway.

Rumen NH_3 concentration reflects the balance between production (mostly from protein degradation) and the summation of microbial utilization 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 improving duodenal N flow, which suggest an improvement in microbial N usage (Balcells et al., 2012). However, the fact that differences in NH_3 concentration have a minor relevance *in vitro* suggests that the recorded differences *in vivo* may be explained though differences in absorption mechanism.

Bioflavex was able to partially buffer rumen pH. This seems to be clearly related with the drop in lactate concentration with BF, although this effect was not consistently supported by

the recorded values in rumen VFA concentration *in vivo* Rumen pH values are primarily determined by fermentation end-products (i.e. VFA, lactate, NH_3; (Owens et al., 1998), where VFA concentration only explains 32 % of the total observed variation in rumen pH (Sauvant et al., 1999) and changes in feed intake, saliva production, dietary buffers, and rate of passage may alter such relationship. In the *in vitro* trial, the bottles supplemented with flavonoids showed a lower numerical VFA concentration but differences did not reach significance.

In both trials, BF supplementation consistently enhanced rumen molar proportion of propionate at the expense of acetate, and thus reduced the acetate-to-propionate ratio. In this sense, it is necessary to remark that rumen concentration and molar proportion of VFAs are indexes of the equilibrium between production and absorption of the end product. However, activity of BF and its components on molar proportions of propionate and acetate was confirmed in the *in vitro* trial, where the absorption process is excluded. Tissues use propionate more efficiently than acetate, and besides propionate may enter in the citric acid cycle in order to produce oxaloacetate, which can then be used to produce glucose via gluconeogenesis. However, it has been demonstrated that differences in rumen fermentation may have a small effect on performances of animals fed high concentrate diets (Schelling, 1984).

In both *in* vivo and *in* vitro trials, supplementation with BF and the flavonoid compounds changed the VFA fermentation profile, which suggests that flavonoids might have modified the rumen microbiota. This has been confirmed in the human gut, where genistein is able to modify prevalent species of microbiota (Clavel et al., 2005; Schoefer et al., 2002). The specific effect of flavonoids has also been demonstrated in the rumen ecosystem, such as that of catequin 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 ciliate

protozoa and archaeas such as hydrogenotrophic methanogens and *Methanosarcina ssp.* (Seradj et al., 2014a). In our study, the inclusion of BF in diet did not alter the rumen abundance of lactate-producing bacteria such as *S. bovis,* but tended improved that of the lactate-consuming species *M. elsdenii* ($P = 0.06$) and *S. ruminantium* ($P < 0.01$), although no changes in abundance of *S. ruminantium* were previously detected (Balcells et al., 2012). Differences in the experimental protocol and the acidity level in the media may probably explain this discrepancy.

The *in vitro* trial support results from *in vivo:* addition of BF improved 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 homogenous, and BF and its main components improved its concentration *in vitro*.

A negative relationship between rumen pH values and lactate concentrations was observed in the *in vivo* trial, pH decreasing after concentrate feeding according to lactate accumulation (Owens et al., 1998). However, only numerical differences in lactate concentration were detected in CTR bottles when the effect of BF and its components were tested *in vitro*, suggesting that lactate absorption under sub-acute acidosis conditions may plays a crucial role in lactate accumulation *in vivo*. Nevertheless, a different behavior in lactate metabolism between the *in vivo* and *in vitro* could not be discarded. Lactate accumulation during acidosis induction may vary largely, exceeding to 50 mmol/L in acute acidosis (Nagaraja et al., 1985) although values lower than 10 mmol/L have been described during subacute acidosis conditions (Balcells et al., 2012; Burrin and Britton, 1986; Goad et al., 1998) or even cases in which concentration was unaffected (Bevans et al., 2005). In our experiment, when sub-acute pH conditions were reached after feeding, a significant lactate accumulation was detected.

Moreover, during acidosis challenge S. *bovis* titters increased, its 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 recorded with BF was confirmed *in vitro,* where a positive effect of BF and its main components on the lactate-consuming bacteria titters was evidenced.

In summary, BF supplementation in high concentrate fed calves was effective in preventing pH reduction and enhancing rumen fermentation efficiency through modifying the activity of lactating-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 might have a role in activity of rumen microbiota.

LITERATURE CITED

AOAC. 1990. Official Methods of Analysis. 15th ed. Assoc. Off. Anal.Chem., Arlington, VA.

Balcells, J., A. Aris, A. Serrano, A. R. Seradj, J. Crespo, and M. Devant. 2012. Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. J. Anim. Sci. 90: 4975-4984.

Beauchemin, K. A., and J. G. Buchanan-Smith. 1990. Effects of Fiber Source and Method of Feeding on Chewing Activities, Digestive Function, and Productivity of Dairy Cows. J. Dairy Sci. 73: 749-762.

Beauchemin, K. A., W. Z. Yang, and L. M. Rode. 2001. Effects of barley grain processing on the site and extent of digestion of beef feedlot finishing diets. J. Anim. Sci. 79: 1925-1936.

334 Bevans, D. W., K. A. Beauchemin, K. S. Schwartzkopf-Genswein, J. J. McKinnon, and T. A.
335 McAllister. 2005. Effect of rapid or gradual grain adaptation on subacute acidosis and
336 feed intake by feedlot cattle. J. Anim. Sci. 83: 1116-1132.
337 Bodas, R., N. Prieto, R. García-González, S. Andrés, F. J. Giráldez, and S. López. 2012.
338 Manipulation of rumen fermentation and methane production with plant secondary
339 metabolites. Anim. Feed Sci. Tech. 176: 78-93.
340 Broudiscou, L. P., and B. Lassalas. 2000. Effects of Lavandula officinalis and Equisetum
341 arvense dry extracts and isoquercitrin on the fermentation of diets varying in forage
342 contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40: 431-440.
343 Broudiscou, L. P., Y. Papon, and A. F. Broudiscou. 2002. Effects of dry plant extracts on
344 feed degradation and the production of rumen microbial biomass in a dual outflow
345 fermenter. Anim. Feed Sci. Tech. 101: 183-189.
346 Burrin, D. G., and R. A. Britton. 1986. Response to monensin in cattle during subacute
347 acidosis. J. Anim. Sci. 63: 888-893.
348 Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and
349 ammonia. Clin. Chem. 8: 130-132.
350 Clavel, T., G. Henderson, C. A. Alpert, C. Philippe, L. Rigottier-Gois, J. Doré, and M. Blaut.
351 2005. Intestinal bacterial communities that produce active estrogen-like compounds
352 enterodiol and enterolactone in humans. Appl. Environ. Microb. 71: 6077-6085.
353 European Communities. 2003. Regulation EC No 1831/2003 of the European Parliament and
354 Council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur.
355 Union L268: 29-43.
356 Gibson, G. R., E. R. Beatty, X. Wang, and J. H. Cummings. 1995. Selective stimulation of
357 bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108:
358 975-982.

359 Gladine, C., E. Rock, C. Morand, D. Bauchart, and D. Durand. 2007. Bioavailability and
360 antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose,
361 in sheep made highly susceptible to lipoperoxidation. Brit. J. Nutr. 98: 691-701.
362 Goad, D. W., C. L. Goad, and T. G. Nagaraja. 1998. Ruminal Microbial and Fermentative
363 Changes Associated with Experimentally Induced Subacute Acidosis in Steers. J.
364 Anim. Sci. 76: 234-241.
365 Jouany, J. P. 1982. Volatile fatty acid and alcohol determination in digestive contents, silage
366 juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments. 2: 131-144.
367 Koenig, K. M., K. A. Beauchemin, and L. M. Rode. 2003. Effect of grain processing and
368 silage on microbial protein synthesis and nutrient digestibility in beef cattle fed
369 barley-based diets. J. Anim. Sci. 81: 1057-1067.
370 Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using
371 real-time quantitative PCR and the 2 -ΔΔCT method. Methods 25: 402-408.
372 Maeda, H., C. Fujimoto, Y. Haruki, T. Maeda, S. Kokeguchi, M. Petelin, H. Arai, I.
373 Tanimoto, F. Nishimura, and S. Takashiba. 2003. Quantitative real-time PCR using
374 TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans,
375 Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. FEMS
376 Immunol. Med. Mic. 39: 81-86.
377 Mould, F. L., R. Morgan, K. E. Kliem, and E. Krystallidou. 2005. A review and
378 simplification of the in vitro incubation medium. Anim. Feed Sci. Tech. 123-124 Part
379 1: 155-172.
380 Nagaraja, T. G., T. B. Avery, S. J. Galitzer, and D. L. Harmon. 1985. Effect of ionophore
381 antibiotics on experimentally induced lactic acidosis in cattle. Am. J. Vet. Res. 46:
382 2444-2452.

383 Ouwerkerk, D., A. V. Klieve, and R. J. Forster. 2002. Enumeration of Megasphaera elsdenii
384 in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92: 753-758.
385 Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in Cattle: A Review. J.
386 Anim. Sci. 76: 275-286.
387 Patra, A. K., and J. Saxena. 2009. Dietary phytochemicals as rumen modifiers: A review of
388 the effects on microbial populations. Anton. Leeuw. Int. J. G. 96: 363-375.
389 Rhodes, M. J. C. 1996. Physiologically-active compounds in plant foods: An overview. P.
390 Nutr. Soc. 55: 371-384.
391 Sauvant, D., F. Meschy, and D. Mertens. 1999. Les composantes de l'acidose ruminale et les
392 effets acidogènes des rations. INRA Prod. Anim. 12: 49-60.
393 Schelling, G. T. 1984. Monensin mode of action in the rumen. J. Anim. Sci. 58: 1518-1527.
394 Schoefer, L., R. Mohan, A. Braune, M. Birringer, and M. Blaut. 2002. Anaerobic C-ring
395 cleavage of genistein and daidzein by Eubacterium ramulus. FEMS Microbiol. Lett.
396 208: 197-202.
397 Seradj, A. R., J. Crespo, M. Fondevila, and J. Balcells. 2014a. The effect of a citrus extract
398 rich in flavonoids (Bioflavex) and its main components on rumen fermentation and
399 microbial population under in vitro system using steers fed high concentrate diet as
400 rumen liquor donors Joint Annual Meeting, Kansas City, US.
401 Seradj, A. R., J. Crespo, D. Villalba, and J. Balcells. 2014b. Inhibition of rumen
402 methanogenesis induced by Bioflavex and its pure flavonoid components under in
403 vitro fermentation using rumen fluid from steers fed high concentrate diets Joint
404 Annual Meeting. ADSA-ASAS, Kansas City, US.
405 Stack, R. J., R. E. Hungate, and W. P. Opsahl. 1983. Phenylacetic acid stimulation of
406 cellulose digestion by Ruminococcus albus 8. Appl. Environ. Microb. 46: 539-544.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-Dependent Shifts in the Bacterial Population of the Rumen Revealed with Real-Time PCR. Appl. Environ. Microb. 67: 2766-2774.

Taylor, K. A. C. C. 1996. A simple colorimetric assay for muramic acid and lactic acid. Appl. Biochem. Biotechnol. Enzym. Eng. Biotechnol. 56: 49-58.

Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48: 185-197.

Tzounis, X., J. Vulevic, G. G. C. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. Kwik-Uribe, and J. P. E. Spencer. 2008. Flavanol monomer-induced changes to the human faecal microflora. Brit. J. Nutr. 99: 782-792.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Winter, J., L. H. Moore, V. R. Dowell Jr, and V. D. Bokkenheuser. 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microb. 55: 1203-1208.

Wu, V. C. H., X. Qiu, B. G. de los Reyes, C. S. Lin, and Y. Pan. 2009. Application of cranberry concentrate (Vaccinium macrocarpon) to control Escherichia coli O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa. Food Microbiol. 26: 32-38.

429 Table 1. Ingredients (%) of the concentrate mixture and chemical composition (g/kg DM) of
430 concentrate and straw used.

Item (%)	Diets	
	CTR	BF
Barley straw		
Concentrate		
Corn grain	35	35
Barley grain	25	25
Soybean Meal (44 %)	10	10
Wheat Bran	6	6
Sunflower meal (30 %)	3.5	3.5
Gluten Feed (20 %)	8	8
Beet Pulp	7	7
Palm oil	2.5	2.5
Calcium	1.3	1.3
bi-calcium phosphate	0.8	0.8
Salt	0.3	0.3
Mineral and vitamin premix	0.4	0.4
Sepiolite	0.2	0.2
Bioflavex (mg/kg)	0	450
Chemical Composition (g/kg DM)		
	Concentrate	Barley Straw
DM	90.61	89.04
OM	93.16	93.35
CP	15.43	3.78
NDF	29.26	74.41

431 Mineral and vitamin premix, [IU /kg]: Vitamin A, 5.000; Vitamin D3, 800; [mg/kg]; Vitamin
432 E, 12; Zn (from zinc oxide), 80; Se (from sodium selenate), 0.15; Co (from cobalt
433 Carbonate), 0.2; Mg (from magnesium oxide), 32; Cu (from copper sulfate), 3.18; Fe (from
434 Ferrous Carbonate), 24; K (from Potassium iodide), 0.4.

435 Table 2. Specific primer sets used in the Experiment.

Target	Author	Primers	
		Forward	Reverse
Quantitative PCR			
†Total bacteria	(Maeda et al., 2003)	5′-GTGSTGCAYGGYTGTCGTCA-3′	5′-ACGTCRTCCMCACCTTCCCC-3′
†Selenomonas ruminantium	(Tajima et al., 2001)	5′-TGCTAATACCGAATGTTG-3′	5′-TCCTGCACTCAAGAAAGA-3′
†Streptococcus bovis	(Tajima et al., 2001)	5′-CTAATACCGCATAACAGCAT-3′	5′-AGAAACTTCCTATCTCTAGG-3′
†Megasphaera elsdenii	(Ouwerkerk et al., 2002)	5′-GACCGAAACTGCGATGCTAGA-3′	5′-CGCCTCAGCGTCAGTTGTC-3′

436 †qPCR conditions: 1 x (95 º C 10:00 min), 40 x (95 º C 00:15 min, 60 º C 00:10 min, 72 º C

437 00:55 min) + Melt Curve

438 Efficiency for all the used primes were between 90 to 95 %

439

440 Table 3: Pattern of rumen fermentation parameters in intensively fed growing calves given concentrate with (BF) or without (CTR)

441 the flavonoid mixture (Bioflavex)

Items	Treatments		SEM	Hours			SEM	*P* value[1]		
	CTR	BF		0	4	8		Tr	H	Tr x H
pH	5.8	6.1	0.05	6.9	5.4	5.5	0.06	<.01	<.01	0.37
NH3, mg /L	28.0	14.7	2.80	40.0	8.4	15.7	3.30	<.01	<.01	0.17
VFA, mM	92.1	96.7	3.84	60.6	113.8	108.7	4.53	0.41	<.01	0.37
VFA, mol/100 mol										
Acetate	61.9	60.6	0.68	64.5	60.1	59.3	0.80	0.19	<.01	0.01
Propionate	22.5	24.2	0.60	21.0	24.4	24.7	0.71	0.05	<.01	<.01
Butyrate	10.7	10.2	0.44	9.0	10.9	11.4	0.52	0.44	<.01	0.92
Iso-Butyrate	1.0	1.0	0.05	1.29	0.89	0.81	0.05	0.89	<.01	0.90
Valerate	2.7	2.6	0.12	2.46	2.65	2.81	0.14	0.36	0.20	0.41
Iso-Valerate	1.1	1.3	0.11	1.71	1.01	0.97	0.132	0.15	<.01	0.75

442 Bioflavex (BF; Interquim S. A. (FerrerHealthTech), Sant Cugat, Barcelona, Spain) and Control (CTR)

443 [1]Tr = treatment effect; H = time post feeding; Tr x H = interaction of the treatment and time post feeding

444 Table 4 Effects of Bioflavex on rumen lactate concentrations (mmol/L), absolute and relative quantification of *Streptococcus bovis*,

445 *Selenomonas ruminantium*, *Megasphaera elsdenii* and over the post feeding hours.

Items	Treatments		SEM	Hours			SEM	*P* value[1]		
	CTR	BF		0	4	8		Tr	H	Tr x H
Lactate, mmol/L	2.08	0.91	0.109	0.81	1.71	1.83	0.38	<.01	<0.01	0.22
Relative Quantification										
S. bovis	0.10	0.09	0.007	0.12	0.09	0.08	0.009	0.80	0.016	0.96
M. elsdenii	0.02	0.05	0.011	0.01	0.04	0.06	0.013	0.06	0.02	0.19
S. ruminantium	2.4	3.3	0.19	2.9	2.8	2.9	0.22	<.01	0.99	0.06

446 [¥] Log 16s rDNA copy num/g FM

447 [†] $2^{(-\Delta Ct)} \times 10^2$

448 [1]Tr = treatment effect; H = time post feeding; Tr x H = interaction of the treatment and time post feeding

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

	Treatments									
Time			*BF-Major Components*			*BF-Tracer Components*				
(hour)	CTR	BF	NG	NH	PC	HS	IN	NE	SEM	*P* value
2	47.7^{ab}	39.9^{bc}	25.4^{d}	37.3^{bc}	40.2^{bc}	51.8^{a}	43.3^{abc}	35.9^{c}	2.09	
4	92.5^{ab}	85.8^{bc}	83.2^{bcd}	75.7^{d}	81.5^{cd}	96.9^{a}	85.8^{bc}	89.7^{abc}	1.91	
6	126.8^{abc}	119.8^{cd}	120.0^{bcd}	104.8^{e}	113.3^{de}	130.4^{a}	120.2^{bcd}	128.9^{ab}	1.87	<.01
8	153.2^{ab}	143.5^{cd}	149.6^{bcd}	131.0^{e}	142.0^{d}	151.4^{abc}	147.3^{bcd}	159.5^{a}	1.90	
12	173.8^{ab}	162.7^{c}	166.9^{bc}	149.3^{d}	161.2^{c}	168.8^{abc}	165.1^{bc}	179.1^{a}	2.06	

451 Different superscripts $^{(a,b,c)}$ denote statistical differences ($P < 0.05$) among treatments

452 Bioflavex (BF), Naringine (NG), Neohesperidine (NH), Poncirine (PC), Hesperidine (HS), Isonaringine (IN) , Neoeriocitrine (NE), , and Control
453 (CTR)

454 Table 6. *In vitro* fermentation parameters in response to flavonoid mixture (Bioflavex) and its pure flavonoid components

Items	Treatments								SEM	*P* value
			BF-Major Components			*BF-Tracer Components*				
	CTR	BF	NG	NH	PC	HS	IN	NE		
Parameters										
NH3-N (mg/L)	158.8 [a]	143.8 [ab]	149.0 [ab]	146.1 [ab]	140.5 [ab]	152.5 [ab]	135.9 [b]	150.1 [ab]	10.21	0.04
VFA, mM	37.5	34.7	31.9	33.4	33.4	33.4	33	34.6	2.97	0.43
VFA, mol/100 mol										
Acetate	58.9 [a]	53.6 [b]	51.3 [b]	52.0 [b]	52.5 [b]	53.2 [b]	51.5 [b]	52.2 [b]	1.68	<.01
Propionate	25.5 [b]	30.3 [a]	33.0 [a]	31.9 [a]	32.4 [a]	30.7 [a]	33.7 [a]	31.8 [a]	1.94	<.01
Butyrate	10.2	10.3	9.8	10.4	9.7	10.6	9.4	10.1	0.69	0.9
Iso-Butyrate	1.9	2	2	2	1.8	1.9	1.7	2	0.21	0.3
Valerate	2	2.1	2.1	2.1	2.1	2.1	2.2	2.3	0.13	0.33
Iso-Valerate	1.5	1.7	1.7	1.7	1.6	1.7	1.6	1.7	0.35	0.9

455 Different superscripts [(a,b,c)] denote statistical differences ($P < 0.05$) among treatments

456 Bioflavex (BF), Naringine (NG), Neohesperidine (NH), Poncirine (PC), Hesperidine (HS), Isonaringine (IN) , Neoeriocitrine (NE), , and Control

457 (CTR)

458 Table 7. Effects of Bioflavex on lactate concentration (mmol/L), absolute quantification of total bacteria and relative quantification of
459 *Streptococcus bovis*, *Selenomonas ruminantium*, *Megasphaera elsdenii* over the incubation time.

Items	Treatments									
	CTR	BF	*BF-Major Components*			*BF-Tracer Components*			SEM	*P* value
			NG	NH	PC	HS	IN	NE		
Lactate, mmol/L	0.81	0.45	0.55	0.5	0.98	0.48	1.02	1.35	0.07	0.1
Relative Quantification										
S. bovis[†]	0.015 ^{b}	0.014 ^{b}	0.013 ^{bc}	0.002 ^{d}	0.005 ^{cd}	0.014 ^{b}	0.005 ^{cd}	0.032 ^{a}	0.0015	<.01
M. elsdenii[‡]	0.8 ^{b}	4.9 ^{a}	4.4 ^{ab}	5.7 ^{a}	3.1 ^{ab}	1.1 ^{b}	3.6 ^{ab}	1.3 ^{b}	0.67	<.01
S. ruminantium[†]	0.1 ^{b}	0.5 ^{ab}	0.4 ^{ab}	0.2 ^{ab}	0.4 ^{ab}	0.6 ^{a}	0.5 ^{ab}	0.1 ^{b}	0.08	0.01

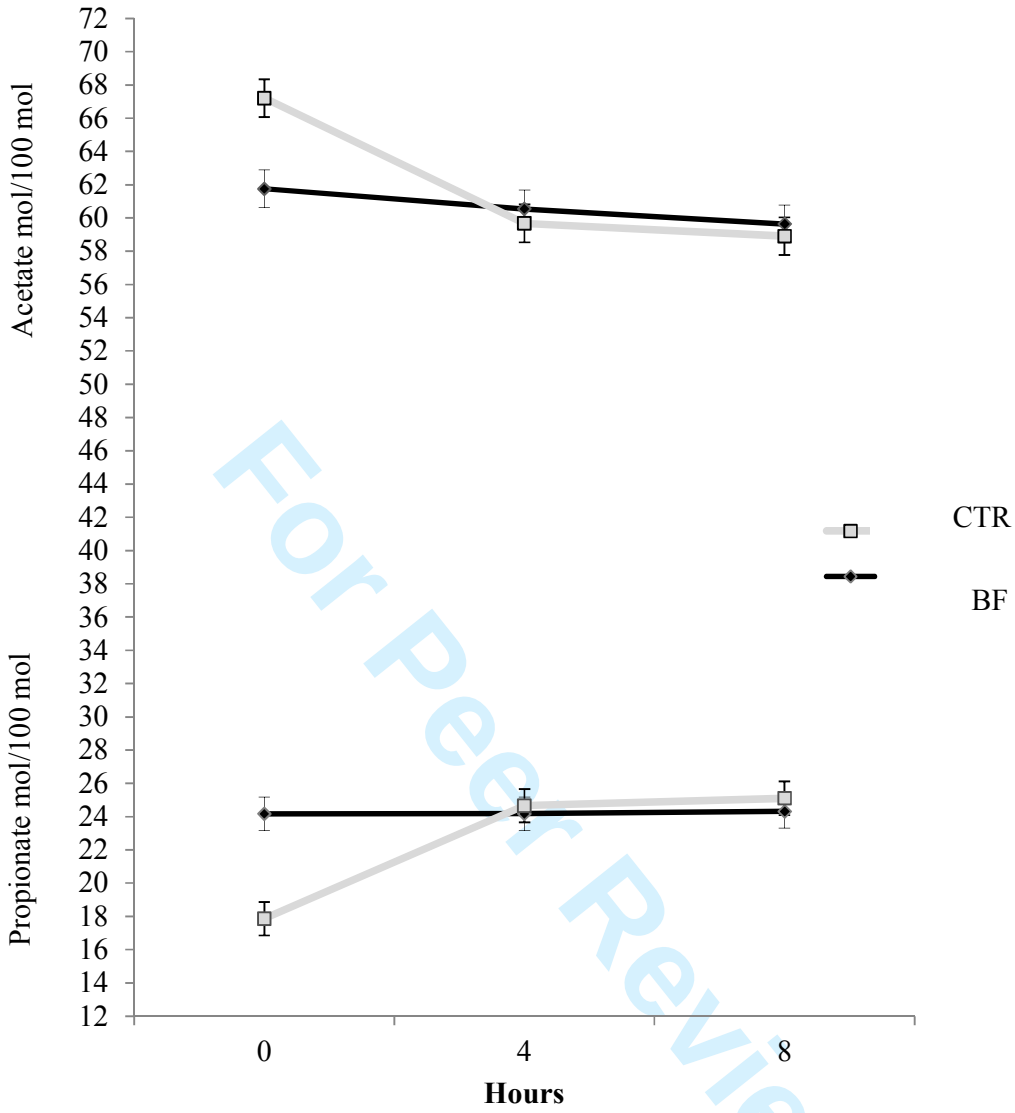
460 [¥] Log 16s rRNA copy num/g FM

461 [†] $2^{(-\Delta Ct)} \times 10^{2}$

462 [‡] $2^{(-\Delta Ct)} \times 10^{6}$

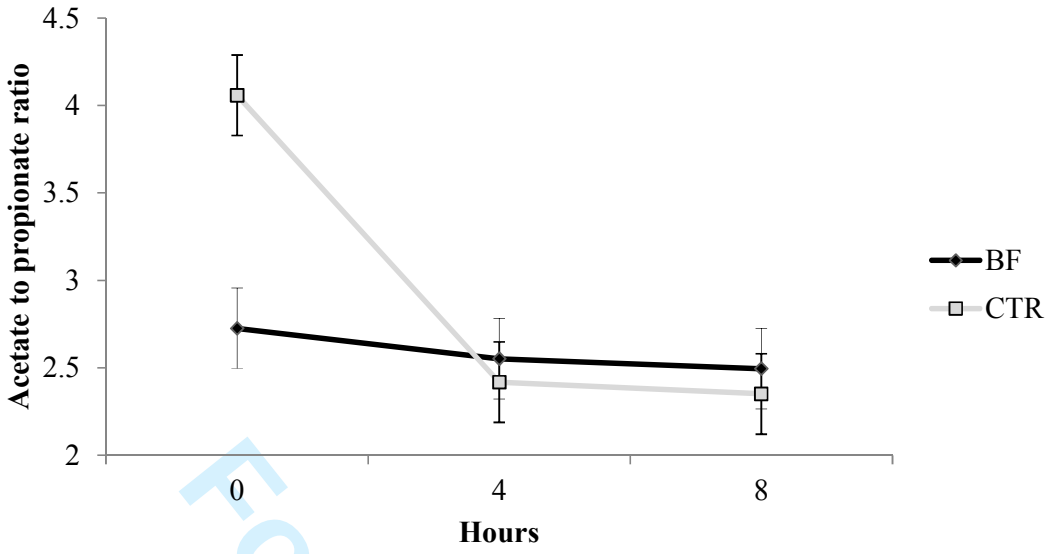
463 Different superscripts $^{(a,b,c)}$ denote statistical differences ($P < 0.05$) among treatments

464 Bioflavex (BF), Naringine (NG), Neohesperidine (NH), Poncirine (PC), Hesperidine (HS), Isonaringine (IN) , Neoeriocitrine (NE), , and Control
465 (CTR)



466

467 a



468 b.

469 **Figure 1.** Changes in molar proportion of acetate (a) and propionate (b) registered in
470 response to a commercial flavonoid supplement (Bioflavex; Interquim S.A.
471 (FerrerHealthTech), Sant Cugat, Barcelona, Spain) in 8 rumen-cannulated steers in relation to
472 time post feeding (interaction of treatment × time post feeding).

Animal Feed Science and Technology 197 (2014) 85–91

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

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

A.R. Seradj[a], L. Abecia[b], J. Crespo[c], D. Villalba[a], M. Fondevila[d], J. Balcells[a,*]

[a] Departament Producció Animal, ETSEA, Agrotecnio Center, Alcalde Rovira Roure 191, 25198 Lleida, Spain
[b] Animal Nutrition Institute, Estación Experimental del Zaidín (CSIC), Profesor Albareda 1, 18008 Granada, Spain
[c] Interquim S. A. (Ferrer Health Tech), Sant Cugat, Barcelona, Spain
[d] Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 25 February 2014
Received in revised form 26 August 2014
Accepted 27 August 2014

Keywords:
Flavonoids
In vitro incubation
Gas production
Methanogenesis

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 μg/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 propionate 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.

Abbreviations: BF, Bioflavex®; CP, crude protein; CTR, control; DM, dry matter; HMA, hydrogenotrophic methanogenic archaea; HS, hesperidine; IN, isonaringine; NDF, neutral detergent fiber; NE, neoeriocitrine; NG, naringine; NH, neohesperidine; PC, poncirine; qPCR, quantitative polymerase chain reaction; TA, total archaea.

* Corresponding author. Tel.: +34 97 370 64 98; fax: +34 973702874.
E-mail addresses: balcells@prodan.udl.cat, qbalcells@hotmail.com (J. Balcells).

http://dx.doi.org/10.1016/j.anifeedsci.2014.08.013
0377-8401/

1. 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.

2. Materials and methods

2.1. Incubation procedure

The effect of Bioflavex® (BF) and its pure flavonoid components (Hesperidine, HS; Isonaringine, IN; Naringine, NG; Neoeriocitrine, NE; Neohesperidine, NH and Poncirine, PC) on rumen fermentation were tested against the control (CTR) at 200 μg/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 inoculum 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

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.

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″/2 L, Hamilton syringe Gastight®, Nevada, USA) and immediately analyzed for methane concentration.

2.2. 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 μm, 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.

2.3. 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 $2^{-\Delta Ct}$ (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.

Table 2
Specific primer sets for qPCR used in the Experiment.

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

† 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).

2.4. 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.

3. 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 consuming 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).

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).

4. 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

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).
† $2^{(-\Delta Ct)} \times 10^{2}$.
‡ $2^{(-\Delta Ct)} \times 10^{6}$.

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 depression 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.

5. 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.

Acknowledgements

This research was financed by Interquim S. A. (Ferrer Health Tech), Sant Cugat, Barcelona, Spain.

The authors would like to thank the staff of the Animal Nutrition lab and Dr. M. Llovera and Dr. T. Capell for their co-operation.

References

AOAC, 2005. In: Horwitz, W., Latimer, G.W. (Eds.), Official Methods of Analysis. , 18th ed. Association of Official Analytical Chemists, Gaithersburg, MA, USA.

Balcells, J., Aris, A., Serrano, A., Seradj, A.R., Crespo, J., Devant, M., 2012. Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. J. Anim. Sci. 90, 4975–4984.

Beuvink, J., Spoelstra, S., 1992. Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro. Appl. Microbiol. Biotechnol. 37, 505–509.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F.J., López, S., 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Technol. 176, 78–93.

Broudiscou, L.-P., Papon, Y., Broudiscou, A.F., 2000. Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes. Anim. Feed Sci. Technol. 87, 263–277.

Broudiscou, L.P., Lassalas, B., 2000. Effects of Lavandula officinalis and Equisetum arvense dry extracts and isoquercitrin on the fermentation of diets varying in forage contents by rumen microorganisms in batch culture. Reprod. Nutr. Dev. 40, 431–440.
Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8, 130–132.
Chen, M., Wolin, M.J., 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microb. 38, 72–77.
Cushnie, T.P.T., Lamb, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents 38, 99–107.
De Freitas, J.A., De Souza, J.C., Lana, R.P., Antonangelo, R.P., De Freitas, A.A., De Santana, R.T.S., 2007. Ethanolic extract of propolis in lactating cows. J. Dairy Sci. 90 (suppl. 1), 105.
Demeyer, D.I., Van Nevel, C.J., 1975. Methanogenesis, an integrated part of carbohydrate fermentation, and its control. In: Digestion and Metabolism in the Ruminant., pp. 366–382.
Denman, S.E., Tomkins, N.W., McSweeney, C.S., 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol. Ecol. 62, 313–322.
Franke-Whittle, I.H., Goberna, M., Insam, H., 2009. Design and testing of real-time PCR primers for the quantification of Methanoculleus, Methanosarcina, Methanothermobacter, and a group of uncultured methanogens. Can. J. Microbiol. 55, 611–616.
Garcia-Lopez, P.M., Kung, L., Odom, J.M., 1996. In vitro inhibition of microbial methane production by 9,10-anthraquinone. J. Anim. Sci. 74, 2276–2284.
Gladine, C., Rock, E., Morand, C., Bauchart, D., Durand, D., 2007. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Br. J. Nutr. 98, 691–701.
Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.
Jouany, J.P., 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments 2, 131–144.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.
Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., Takashiba, S., 2003. Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. FEMS Immunol. Med. Microbiol. 39, 81–86.
McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48, 7–13.
McDonald, I., 1981. A revised model for the estimation of protein degradability in the rumen. J. Agric. Sci. Camb. 96, 251–256.
Mirzoeva, O.K., Grishanin, R.N., Calder, P.C., 1997. Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. Microbiol. Res. 152, 239–246.
Mould, F.L., Morgan, R., Kliem, K.E., Krystallidou, E., 2005. A review and simplification of the *in vitro* incubation medium. Anim. Feed Sci. Technol. 123-124, 155–172.
Nagaraja, T.G., Titgemeyer, E.C., 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. J. Dairy Sci. 90 (Suppl. 1), E17–E38.
Ørskov, E.R., Flatt, W.P., Moe, P.W., 1968. Fermentation balance approach to estimate extent of fermentation and efficiency of volatile fatty acid formation in ruminants. J. Dairy Sci. 51, 1429–1435.
Oskoueian, E., Abdullah, N., Oskoueian, A., 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. BioMed Res. Int. 2013, 8.
Ouwerkerk, D., Klieve, A.V., Forster, R.J., 2002. Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92, 753–758.
Øvreås, L., Torsvik, V., 1998. Microbial diversity and community structure in two different agricultural soil communities. Microb. Ecol. 36, 303–315.
Patra, A.K., Saxena, J., 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71, 1198–1222.
Rhodes, M.J.C., 1996. Physiologically-active compounds in plant foods: an overview. Proc. Nutr. Soc. 55, 371–384.
Stack, R.J., Hungate, R.E., Opsahl, W.P., 1983. Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8. Appl. Environ. Microbiol. 46, 539–544.
Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y., 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 67, 2766–2774.
Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48, 185–197.
Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.
Wang, D., Huang, J., Zhang, Z., Tian, X., Huang, H., Yu, Y., Zhang, G., Ding, J., Huang, R., 2013. Influences of Portulaca oleracea extracts on in vitro methane emissions and rumen fermentation of forage. J. Food Agric. Environ. 11, 483–488.
Winter, J., Moore, L.H., Dowell Jr., V.R., Bokkenheuser, V.D., 1989. C-ring cleavage of flavonoids by human intestinal bacteria. Appl. Environ. Microbiol. 55, 1203–1208.
Yaghoubi, S.M.J., Ghorbani, G.R., Rahmani, H.R., Nikkhah, A., 2007. In vitro manipulation of rumen fermentation by propolis flavonoids and monensin. J. Dairy Sci. 90 (suppl. 1), 105–106.
Yáñez-Ruiz, D.R., Macías, B., Pinloche, E., Newbold, C.J., 2010. The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. FEMS Microbiol. Ecol. 72, 272–278.