Universitat Autònoma de Barcelona

Effects of the supplementation with citrus flavonoids on performance, eating pattern and animal behavior in fattening cattle

Montserrat Paniagua Jiménez

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Thesis

Presented to the Animal and Food Science Department of Veterinary

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In Partial Fulfillment of the Requirements for the Degree of

DOCTOR IN ANIMAL PRODUCTION

By

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Directed by

Maria Devant Guille

Bellaterra, September 2019

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MARIA DEVANT GUILLE, investigadora del Programa de Remugants de l'Institut de Recerca i Tecnologia Agroalimentàries, i

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Que la memòria titulada "**Effects of the supplementation with citrus flavonoids on performance, eating pattern and animal behavior in fattening cattle**", presentada per Montserrat Paniagua Jiménez per optar al grau de Doctor en Producció Animal per la Universitat Autònoma de Barcelona, ha estat realitzada sota la direcció de la Dra. Maria Devant Guille i, considerant-la acabada, autoritzen la seva presentació per tal que sigui jutjada per la comissió corresponent.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, 19 de septembre de 2019.

Maria Devant Guille	**Sergio Calsamiglia Blancafort**
Directora	**Tutor**

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ABSTRACT

Nowadays, improving productivity, health and animal welfare in livestock reared under intensive conditions is mandatory for the sustainability of our production systems. Furthermore, reducing the use of antibiotics due to the constant growth of resistant bacteria is a critical issue to face. Consequently, the research and development of new proposals beyond traditional solutions applied are essential, and probably also a new approach of animal metabolism, health and well-being is needed to tackle all these important challenges.

Citrus flavonoids are polyphenols that possess interesting anti-inflammatory, antioxidant, and antimicrobial properties and have showed promising effects in previous research on beef cattle. Otherwise, recently the knowledge of a communication network between gastrointestinal tract, microbiota, and the central nervous system, the gut-brain axis, has increased. Thus, inflammation, microbiota, and diet have been proposed to be involved in animal behavior and eating pattern modulation.

Therefore, the main objective of this thesis was to study the possible benefits of supplementing citrus flavonoids in bulls fattened under intensive conditions and fed high-concentrate diets on performance and productivity, but also their possible effects on eating and animal behavior and to study the possible mode of action related with the gut-brain axis.

Thus, a first study evaluated the effects of citrus flavonoids supplementation on eating pattern (using a single-space feeder), performance parameters, rumen wall health, and animal behavior in Holstein bulls fed high-concentrate diets in pellet form. Citrus flavonoids reduced feed intake and modified eating pattern, reducing the percentage of large meal sizes. Additionally, rumen wall health and animal behavior were improved. Based on these results, the following studies evaluated the possible interactions of citrus flavonoids supplementation with feeder space, concentrate presentation (pellet vs. meal) and composition (fat level). Furthermore, the expression of genes involved in the gut-brain axis crosstalk, such as nutrient sensing receptors, some neurotransmitters receptors and different inflammation regulators were studied in the epithelium of rumen and, in the last study, of duodenum epithelium as well.

When bulls were supplemented with citrus flavonoids devoted more time to perform feeding behaviors throughout the different studies. Whilst performance parameters and concentrate intake were not affected when concentrate was fed in pellet form using multi-space feeders, concentrate intake was reduced when citrus flavonoids were supplemented in a concentrate fed in meal form without impairing performance, so improving efficiency. In all studies, animal behavior was also improved, by reducing oral non-nutritive behaviors, and aggressive and sexual interactions. Moreover, in all studies, the macroscopical rumen wall study performed at the slaughterhouse showed lighter color for bulls supplemented with citrus flavonoids. Conversely, ruminal gene expression differed among the studies, so the expression of genes involved in nutrient sensing and behavior in bulls supplemented with citrus flavonoids were different depending on the concentrate presentation (pellet or meal), and composition (fat level inclusion). The expression of these genes in the duodenum epithelium when bulls were fed high-fat diets was also modified.

In conclusion, citrus flavonoids supplementation modified eating pattern in Holstein bulls, decreasing the percentage of large meal sizes or increasing time devote to feeding events. This modulation of the eating pattern, together with an improvement in rumen wall might be involved in the improvement of animal behavior of bulls supplemented with citrus flavonoids. Moreover, flavonoid supplementation differently modified the expression of genes in the rumen and duodenum epithelium that could be related with eating pattern and animal behavior regulation, although concentrate presentation (pellet vs. meal) and fat level might affect these effects on gene expression of different nutrient sensing, peptides and hormones receptors, along with some pro-inflammatory molecules, probably as result of the effect of rumen fermentation on flavonoid metabolism.

RESUM

Avui dia millorar la productivitat, la salut i el benestar animal en els sistemes intensius de producció és bàsic per assegurar la seva sostenibilitat. A més, ens enfrontem a la reducció de l'ús d'antibiòtics pel creixement dels bacteris multirresistents. En conseqüència, és essencial investigar i desenvolupar noves alternatives juntament amb un nou enfocament del metabolisme, la salut i el benestar animal necessari per abordar aquests importants desafiaments.

Els flavonoides cítrics són polifenols que posseeixen propietats antiinflamatòries, antioxidants i antimicrobianes, i han mostrat efectes prometedors en estudis previs realitzats en vedells d'engreix. D'altra banda, el creixent coneixement sobre una xarxa de comunicació entre el tracte gastrointestinal, la microbiota i el sistema nerviós central, l'eix intestí-cervell, proposa que la inflamació, la microbiota i la dieta estarien involucrades en la modulació de la conducta animal i alimentària.

Així, l'objectiu d'aquesta tesi va ser estudiar els beneficis de suplementar flavonoides cítrics en vedells d'engreix alimentats amb dietes altes en concentrat sobre la productivitat, la conducta alimentària i animal, aprofundint en els possibles mecanismes d'acció relacionats amb l'eix intestí-cervell.

El primer estudi va avaluar els efectes dels flavonoides cítrics sobre la conducta alimentària (menjadora uniboca), paràmetres productius, salut de la paret ruminal i comportament animal en vedells Holstein alimentats amb concentrat granulat. Els flavonoides van reduir la ingesta de concentrat i van modificar la conducta alimentària, reduint el percentatge de menjades grans. A més, es va millorar la salut de la paret ruminal i el comportament animal. En base a aquests resultats, els següents estudis van avaluar les possibles interaccions de suplementar flavonoides cítrics amb l'espai de menjadora, presentació (granulat vs. farina) i composició (nivell de greix) del pinso. També es va estudiar l'expressió de gens relacionats amb l'eix intestí-cervell, com receptors de nutrients, de neurotransmissors i diferents reguladors de la inflamació en l'epiteli ruminal i, en l'últim estudi, duodenal.

En tots els estudis, els vedells suplementats amb flavonoides van dedicar més temps als comportaments alimentaris. Si bé els paràmetres productius i la ingesta de concentrat no es van veure afectats quan el concentrat es va subministrar granulat

utilitzant menjadores multiespai, els flavonoides van reduir la ingesta de concentrat quan es va subministrar en farina, sense afectar el rendiment i millorant l'eficiència. En els diferents estudis, el comportament animal també va millorar, reduint comportaments orals no nutritius i les interaccions agressives i sexuals. A més, l'estudi macroscòpic de la paret ruminal realitzat en escorxador va mostrar un color més clar en els vedells suplementats amb flavonoides en els diferents estudis. Per contra, l'expressió gènica en l'epiteli ruminal va ser diferent entre estudis, sent l'expressió de gens relacionats amb la detecció de nutrients i el comportament diferent depenent de la presentació del concentrat (pellet o farina) i la seva composició (nivell de greix) en vedells suplementats amb flavonoides cítrics. L'expressió d'aquests gens en l'epiteli duodenal també va ser modificada en vedells alimentats amb dietes altes en greix.

En conclusió, la suplementació amb flavonoides cítrics va modificar la conducta alimentària en els vedells, disminuint el percentatge de menjars grans o augmentant el temps dedicat comportaments alimentaris. Aquesta modulació de la conducta alimentària, juntament amb la salut de la paret ruminal podria explicar la millora del comportament en aquests vedells. A més, la suplementació amb flavonoides va modificar l'expressió de gens en l'epiteli ruminal i duodenal que podrien estar relacionats amb la conducta alimentària i animal. La presentació del concentrat (granulat vs. farina) i el nivell de greix van afectar a aquesta expressió gènica de receptors de nutrients, pèptids i hormones, i de molècules pro-inflamatòries, probablement com a resultat del metabolisme dels flavonoides en el rumen.

RESUMEN

Hoy en día mejorar la productividad, la salud y el bienestar animal en los sistemas intensivos de producción es básico para asegurar su sostenibilidad. Además, nos enfrentamos a la reducción del uso de antibióticos por el crecimiento de bacterias multirresistentes. Consecuentemente, es esencial investigar y desarrollar nuevas alternativas y, posiblemente, un nuevo enfoque del metabolismo, salud y bienestar animal es necesario para abordar estos importantes desafíos.

Los flavonoides cítricos son polifenoles que poseen propiedades antiinflamatorias, antioxidantes y antimicrobianas, y han mostrado efectos prometedores en estudios previos con terneros de cebo. Por otro lado, el creciente conocimiento sobre una red de comunicación entre tracto gastrointestinal, microbiota y sistema nervioso central, el eje intestino-cerebro, propone que inflamación, microbiota y dieta podrían estar involucradas en la modulación de la conducta animal y alimentaria.

Así, el objetivo de esta tesis fue estudiar los beneficios de suplementar flavonoides cítricos en terneros de cebo alimentados con dietas altas en concentrado sobre el rendimiento productivo, la conducta alimentaria y animal, profundizando en los posibles mecanismos de acción relacionados con el eje intestino-cerebro.

El primer estudio evaluó los efectos de suplementar flavonoides cítricos sobre la conducta alimentaria (comedero uniboca), parámetros productivos, salud de la pared ruminal y comportamiento animal en terneros Holstein alimentados con concentrado granulado. Los flavonoides redujeron la ingesta de concentrado y modificaron la conducta alimentaria, reduciendo el porcentaje de comidas grandes. Además, se mejoró la salud de la pared ruminal y el comportamiento animal. En base a estos resultados, los siguientes estudios evaluaron las posibles interacciones de suplementar flavonoides cítricos con el espacio de comedero, presentación (granulado vs. harina) y composición (nivel de grasa) del concentrado. Además, se estudió la expresión de genes relacionados con el eje intestino-cerebro, como receptores de nutrientes, de neurotransmisores y diferentes reguladores de la inflamación en el epitelio ruminal y, en el último estudio, duodenal.

En todos los estudios, los terneros suplementados con flavonoides dedicaron más tiempo a diferentes comportamientos alimentarios. Si bien los parámetros productivos y la ingesta de concentrado no se vieron afectados cuando el concentrado se suministró en

gránulos utilizando comederos multiespacio, los flavonoides redujeron la ingesta de concentrado cuando se suministró en harina, sin afectar el rendimiento y mejorando la eficiencia. En los diferentes estudios, el comportamiento animal también mejoró, reduciendo comportamientos orales no nutritivos y las interacciones agresivas y sexuales. Además, el estudio macroscópico de la pared ruminal realizado en matadero mostró un color más claro en los terneros suplementados con flavonoides cítricos en los diferentes estudios. Por el contrario, la expresión génica en el epitelio ruminal fue diferente entre estudios, siendo la expresión de genes relacionados con la detección de nutrientes y el comportamiento diferente dependiendo de la presentación del concentrado (pellet o harina) y su composición (nivel de grasa) en terneros suplementados con flavonoides cítricos. La expresión de estos genes en el epitelio duodenal también fue modificada en terneros alimentados con dietas altas en grasa.

En conclusión, la suplementación con flavonoides cítricos modificó la conducta alimentaria en los terneros, disminuyendo el porcentaje de comidas grandes o aumentando el tiempo dedicado comportamientos alimentarios. Esta modulación de la conducta alimentaria, junto con la salud de la pared ruminal podría explicar la mejora del comportamiento en estos terneros. Además, la suplementación con flavonoides modificó la expresión de genes en el epitelio ruminal y duodenal que podrían estar relacionados con la conducta alimentaria y animal. La presentación del concentrado (granulado vs. harina) y el nivel de grasa afectaron a esta expresión génica de receptores de nutrientes, péptidos y hormonas, y de moléculas pro-inflamatorias, probablemente como resultado del metabolismo de los flavonoides en el rumen.

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CHAPTER I

Introduction

1. BEEF PRODUCTION SYSTEMS CHALLENGES: SUSTAINABILITY

Nowadays, talking about beef production challenges is synonym of talking about sustainability. The limited resources of our planet along with the climate change and global warming, have placed all our production models in the spotlight, even more when human population is expected to continue growing the next decades, along with an improvement in the standard of life. In fact, worldwide demand for livestock products is predicted to increase twofold by 2050 (Rojas-Downing et al., 2017). On the other hand, climate change will affect both quality and quantity of feed crops, forages, and water availability and these effects will depend on the world area (Moss et al., 2000; Polley et al., 2013; Rojas-Downing et al., 2017). Although during the last decades in the Western world, improvement in knowledge and technologies have allowed an optimization and intensification in livestock production (Napel et al., 2011), unfailingly these systems must be adapted in the coming years to the new climate, environmental conditions and feeds availability, along with developed society demands (Steinfeld et al., 2006; Polley et al., 2013).

The concept of sustainability could be defined, generally speaking, as the capability of satisfying the needs of the present without compromising the capability of the future generations to meet their own needs. The United Nations were already discussing about sustainability in the Johannesburg Declaration on Sustainable Development in 2002 and the three main pillars of sustainability were depicted: economic growth, environmental protection and social equity (Van Cauwenbergh et al., 2007; Boogaard et al., 2011; Hoffmann et al., 2011).

Focusing on intensive livestock production systems, these three main pillars could be adapted as:

- Environmental pillar: the effect of livestock production systems over the climate change. Animal production systems are involved in the emissions of grenhaouse gas (GHG) at different stages, basically due to crops and feed production, farm animal production, manure, processing and transport (Steinfeld et al., 2006; Rojas-Downing et al., 2017).
- Social pillar: includes farmers' quality of life and well-being and also the point of view of the society. Based on the values and concerns of the society,

environmental impact, animal welfare, along with food safety and quality could be also included here (Latruffe et al., 2016).

- Economic pillar: basically profitability and productivity to ensure the economic viability of the farm and the production system (Van Cauwenbergh et al., 2007).

Consequently, sustainability of livestock production systems would imply different meanings depending on the world area and country, its society perceptions and production systems (intensive, extensive, etc.) (Smith et al., 2018).

Regarding beef cattle production, the European Union (EU) is the 3rd largest producer in the world, just after USA and Brazil (Smith et al., 2018), producing 7.8 million tons of bovine meat in 2017 (Eurostat, 2018). European society is characterized by strong ethical concerns about animal welfare and environmental impact of intensive production systems, but either about quality, healthy and nutritive meat consumption (Smith et al., 2018). These society concerns would have been translated into an important legislation on animal welfare and environmental protection, being the EU a worldwide leader in these areas. As a result, European beef industry, and especially farmers, are just facing big challenges that could suppose a deep transformation without precedents in this sector. Thus, the different production systems of beef cattle that coexist in the EU should be adapted to give a proper response to the social and political demands, also maintaining themselves economically viable.

Based on the concept of sustainability, on-farm based challenges of the beef production systems would be defined as:

- ✓ Reducing the environmental impact, basically GHG emissions, being methane the most important GHG in beef cattle due to ruminal fermentation (Steinfeld et al., 2006).
- ✓ Improving on-farm welfare and health.
- ✓ Increasing economic profitability by enhancing farm productivity and animal efficiency.

1.1. Environment impact of beef cattle production

Methane is considered one of the most potent GHG, due to its warming potential per unit, 23 times much than carbon dioxide (Steinfeld et al., 2006). Agriculture is the greatest source of anthropogenic methane emissions and about two-thirds of this methane is produced by enteric fermentation. Globally, total methane released from enteric fermentation is about 86 million tonnes per year, being ruminant livestock one of the most important anthropogenic source of these emissions to the environment (Moss et al., 2000; Steinfeld et al., 2006; Giuburunca et al., 2014). Enteric methane in ruminants is mainly produced in the rumen (87%), and it is the major way to remove hydrogen generated by protozoa, fungi and some bacteria during ruminal anaerobic fermentation of fibrous feeds (Moss et al., 2000; Giuburunca et al., 2014; Cobellis et al., 2016).

Although ruminant species play an important role converting fibrous feeds, basically indigestible for humans, into nutritive products as milk and meat, their environmental impact should be considered and reduced. The production system, feed intake and the quality and digestibility of feed significantly affect rumen methane production and release (IPCC, 1997; Moss et al., 2000). Furthermore, methane production supposes a loss of energy for the animals (between 2 – 15%), so that improving productivity and efficiency of ruminants is the greatest approach to reduce enteric methane emissions (Steinfeld et al., 2006; Giuburunca et al., 2014).

Different nutritional strategies would lead to a reduction of methane production in the rumen. Ruminal fermentation produces different volatile fatty acids (VFA), mainly acetic, propionic and butyric acid, that are used as energy source by ruminants. The quantity of VFA and the molar percentage of the different VFA produced in the rumen are basically determined by the source of feeds, especially by the characteristics and content of carbohydrates. Poor quality feedstuffs with high fibrous content promote acetate production by ruminal microorganisms, whilst in case of feeds with greater quantity and quality of carbohydrates content promote propionate synthesis. Fundamentally, acetate and butyrate boost methane production whereas, on the contrary, propionate is reducing the availability of hydrogen to synthetize methane (Moss et al., 2000). Thus, nutritional strategies focused on enhancing propionate:acetate ratio would reduce ruminal methane production, but this objective will be achieved basically by improving feed efficiency (Knapp et al., 2014). Feeding high-starch diets (increase

propionate production and efficiency), or supplementing with some lipids, as oils, would be nutritional strategies that could lead to a reduction in methane emission by ruminants (knapp et al., 2014).

Beyond nutritional strategies, different alternatives as some feed additives, natural products and extracts, probiotics and ruminal flora modification or vaccination against methanogens have been proposed, achieving different degrees of success (Moss et al., 2000; Bodas et al., 2009; Patra and Saxena, 2010; Seratj et al., 2014; Cobellis et al., 2016).

Additionally, farm management practices focused on improving animal health and reducing mortality, and minimizing metabolic disorders, as heat stress and acidosis, would improve productivity and, consequently, reduce methane emissions per productive unit (Knapp et al., 2014).

1.2. Animal welfare in beef cattle

Animal welfare is actually an outstanding pillar of sustainability of livestock production systems in developed countries. As mentioned before, society values and concerns are clearly influencing animal welfare perception of consumers. During the last half century two important changes have occurred in our developed countries simultaneously: on the one hand, a significant intensification of livestock production systems has taken place; on the other hand, the number of farms and farmers have both decreased, rising at the same time the number of urban people (Boogaard et al., 2011). These urban people possess limited knowledge about farms and livestock production, and their perception about intensive management is quite negative. In fact, their vision of animal welfare is linked with “natural behavior” in a “natural environment”, considering high animal density as a low well-being status (Webster, 2001; Cozzi et al., 2008; Boogaard et al., 2011). Consequently, there is a great interest of farmers to provide an adequate animal welfare to improve the social acceptance of livestock management but also to satisfy consumers’ demands, increasingly influenced by their ethical values.

The absence of chronic stress is one of the most important objectives to improve animal welfare (Möstl and Palme, 2002). Chronic stress trigger the release of glucocorticoid hormones in animals that would negatively affect their growth and immune system (Carroll et al. 2007; Burdick et al., 2011). Carroll et al. (2007) classified

the stressors in livestock in three categories: psychologic stress (associated with fear), physiologic stress (related to endocrine or neuroendocrine function) and physical stress (as heat stress, hunger and thirst, or disease). So, it is highly important to control and reduce these stressors on-farm environment to improve animal welfare and, additionally, to enhance animal productivity and health.

Animal welfare is thereby essential for livestock intensive production systems sustainability, basically due to its relevance on society demands and consumers' decision making, but also for its importance on animal health and productivity either. In fact, all the stakeholders involved, including farmers, legislators and scientist are eager to developed standardized criteria and mechanisms to guarantee an objective animal welfare assessment based on scientific knowledge (McGlone, 2001; Welfare Quality®, 2009). To achieve these objectives, we need to develop doable strategies, easy to apply in practical on-farm conditions but also effective for animal welfare assessment (Webster et al., 2004). Moreover, it is highly important to create the ways to transfer properly this information to the consumers and to the society.

In Europe, a research project named Welfare Quality® was created in 2004, comprising a partnership of 40 institutions from 13 European countries, and expanded in 2006 to 4 more Latin countries, Uruguay, Brazil, Chile and Mexico (Blokhuis et al., 2010). The aim of this multidisciplinary project were to develop standardized and science-based systems for welfare assessment on-farm and slaughterhouses for poultry, pigs and cattle, and to communicate these welfare measures to consumers (Welfare Quality®, 2009; Blokhuis et al., 2010)

The European Welfare Quality® assumes that welfare is a multidimensional concept, which includes physical and mental well-being (Gonyou, 1994). The assessment protocols for fattening cattle, pigs and poultry were published in October of 2009. As a key welfare points four main principles are considered in these protocols: good feeding, good housing, good health and appropriate behavior (Welfare Quality®, 2009). Furthermore, each principle includes two to four independent criteria (Welfare Quality®, 2009). In the **Table 1** we have the principles, criteria and measures that Welfare Quality® protocol for fattening cattle evaluation.

Table 1. The principles, criteria and measures of the Welfare Quality® assessment protocol for fattening cattle.

Welfare Principles	Welfare criteria		Measures
Good feeding	1	Absence of prolonged hunger	Body condition score
	2	Absence of prolonged thirst	Water provision, cleanliness of water points, number of animals using the water points
Good housing	3	Comfort around resting	Time needed to lie down, cleanliness of the animals
	4	Thermal comfort	*As yet, no measure is developed*
	5	Ease of movement	Pen features according to live weight, access to outdoor loafing area or pasture
Good health	6	Absence of injuries	Lameness, integument alterations
	7	Absence of disease	Coughing, nasal discharge, ocular discharge, hampered respiration, diarrhea, bloated rumen, mortality
	8	Absence of pain induced by management procedures	Disbudding/dehorning, tail docking, castration
Appropriate behavior	9	Expression of social behaviors	Agonistic behaviors, cohesive behaviors
	10	Expression of other behaviors	Access to pasture
	11	Good human-animal relationship	Avoidance distance
	12	Positive emotional state	Qualitative behavior assessment

Regarding beef cattle fattened under intensive conditions, it is interesting to highlight the impact of the intensive management in reducing the opportunities for these animals to perform natural behaviors considered as important for ruminants. The impossibility of developing these natural behaviors, frustration and discomfort may generate aggressive interactions and stereotypes, and these are been related with poor welfare status (Gonyou, 1994). Foraging could be considered as a fundamental natural behavior that cannot be accomplished under intensive conditions in beef cattle. In fact, oral stereotypic behaviors are very frequent in these animals (as repetitive licking non-food objects, as fences, walls or the feeder), and have been related with this impossibility to perform natural foraging behavior (Bergeron et al., 2006). On the other hand, talking about bulls reared in intensive fattening systems, aggressive and sexual behaviors frequently performed by these animals suppose a significant welfare challenge (Mach et al., 2009; Devant el al., 2017). The origin of these behaviors is really widespread, as social hierarchy, limited resources (feeder space), anxiety or stress. Testosterone is linked with these behaviors, so castration has been proposed as a possible solution, but this solution would involve negative welfare implications, as it is a mutilation and, additionally, castrated bulls are less efficient affecting carcass quality as well (Mach et al., 2006). Thus,

alternatives to ameliorate and reduce the expression of all these behaviors and improve welfare of beef cattle fattened in intensive production systems are needed.

On the other hand, respiratory disease is considered one of the most important welfare issues in beef cattle reared under intensive conditions (EFSA, 2012). Due to anatomical and physiological factors, and linked to the typical management applied in these productive systems (long transports, mixing of animals, and overstocking), beef cattle are susceptible to a variety of respiratory pathogens that have been summarized under the name of Bovine Respiratory Disease (BRD) (EFSA, 2012). Thus, improving health and reducing the incidence of these BRD are a big challenge to guarantee cattle welfare.

1.3. Efficiency in beef cattle intensive systems

Beef cattle are inherently less efficient than monogatric species, as pigs and poultry, basically due to their digestive system. Although fermentation in the rumen by microorganisms allow ruminants to use low quality diets, with high fibrous content, to produce high quality protein, this process is less efficient when compared with monogastric animals, especially for ruminants reared in extensive grazing systems. Additionally, feed cost is the most important economic factor affecting profitability of beef cattle production, even more when focusing on intensive fattening systems. This cost would count for around 75% of the total direct costs (Nielsen et al., 2013; Kenny et al., 2018). As a consequence, improving feed efficiency is one of the greatest challenges of this industry to guarantee its economic sustainability, reduce environmental impact and improve profitability for the producers, even more when competing with more efficient sources of meat as monogastric livestock.

Generally speaking, efficiency could be defined as the ratio of output per unit of input (Cartens and Tedeschi, 2006; Nielsen et al., 2013). Normally, live body weight and intake are used to measure feed efficiency in livestock, and different measures are commonly employed to calculate this feed efficiency in beef cattle:

- Feed Conversion Ratio (FCR): this is the ratio of intake to live-weight gain, so at lower FCR the more efficient is the animal. These ratio is very valuable to assess feed quality, management and environment, and monitoring beef cattle

performance, because is well correlated to animal growth (Cartens and Tedeschi, 2006; Kenny et al., 2018).

- Feed Conversion Efficiency: is the mathematical inverse of FCR (Kenny et al., 2018), i.e. ratio of live-weight gain to intake. Consequently, regarding feed conversion efficiency the highest the number the more efficient is the animal. As FCR, it is useful to study animal performance, diet quality and management or environment effects in animal efficiency.
- Residual Feed Intake (RFI): this measure uses the expected feed intake of the animal based on its growth and body weight, calculated from intake and performance from a group of contemporary animals (Cartens and Tedeschi, 2006). So, RFI is defined as the difference between the actual feed intake and the expected feed intake based on animal requirements for maintenance and growth (Nielsen et al., 2013; Kenny et al., 2018). This measure is then independent of animals' production level, being more appropriate to study biological mechanisms involved in variation of feed efficiency between beef cattle, but also in programs for genetic improvement of feed efficiency (Cartens and Tedeschi, 2006; Nielsen et al., 2013; Kenny et al., 2018).
- Residual Gain (RG): is the amount of gain adjusted for feed intake (Nielsen et al., 2013). A positive value of RG indicated that the animal is growing faster than predicted based on its intake, so it is more efficient.

Based on these different approaches, genetics and production system are the most relevant factors affecting feed efficiency in beef cattle. Regarding genetic programs, finding and developing genetic predictors for feed efficiency in beef cattle would be highly useful. A key point to improve this efficiency would be to reduce the nutritional requirements for maintenance, where wide animal variation exists (Cartens and Tedeschi, 2006; Nielsen et al., 2013). As previously mentioned, RFI is an interesting measure to select animals with lower energy requirements for maintenance. Additionally, RFI possesses moderate heritability, and some results have suggested that post-weaning RFI in cattle would be a useful trait for selecting to improve efficiency in the progeny (Cartens and Tedeschi, 2006). In fact, actual development of the technology needed to properly study the individual feed intake in beef cattle, facilitates the study of RFI and, consequently, the genetic selection to improve efficiency in cattle.

On the other hand, production system comprising nutrition, management and environment, are greatly affecting feed efficiency in beef cattle as well. Obviously, nutrition is one of the most important of these factors, from the composition of diet to the presentation of feed. It is well-known that high-concentrate diets, with greater content of cereals, energy and higher digestibility are more efficiently used by beef cattle to grow than low quality diets rich in forage of poor quality. Basically, ruminal fermentation of diets rich in fibrous ingredients with low energy content is producing higher acetic acid, and this fermentation is related to a greater production and emission of methane, being less efficient and increasing environmental impact. Conversely, high-concentrate diets based on cereals decreased methane production during ruminal fermentation, also increasing propionic production, being more efficient and reducing environmental impact as well.

Focusing on intensive production systems of beef cattle, a wide range of non-animal factors can affect feed efficiency of livestock. As previously commented, factors affecting rumen fermentation would have an impact on feed efficiency and will modulate the eating pattern of animals at the same time, so different nutritional strategies would impact feed efficiency in beef cattle fed high-concentrate diets, as:

- Concentrate presentation: e.g. feeding the concentrate in pellets improves feed efficiency when compared with meal presentation, basically due to the increase in starch availability as a result of pelleting process, but also by reducing concentrate waste by the animals when eating (Bertipaglia et al., 2010).
- Concentrate composition: some ingredients used in high-concentrate diets, as fats, would have different effects on feed efficiency by modulating feed intake and eating pattern, depending on the inclusion rate but also on its chemical composition (saturated or unsaturated fats) (Krehbiel et al., 2006; Hess et al., 2008).

Additionally, also different feeding strategies could allow us to reduce concentrate intake and waste without impairing performance and animal welfare (as the feeder design and space, or pelleting quality of the concentrate) (Verdú, 2015).

Actually, the type of diet (concentrate, or forage) modulates the way animals eat. Beef cattle are considered as grass and roughage eaters within ruminant species

(Hofmann, 1989), so their digestive system has evolved being adapted to efficiently digest high fibrous foods. Furthermore, foraging seems to be an essential natural behavior for cattle and might be in the origin of oral stereotypes and anxiety in animals reared under intensive conditions (Bergeron et al., 2006). Therefore, it could be hypothesized that a close relationship between eating behavior and animal welfare exists in beef cattle, linking nutrition with behavior (Nielsen, 1999).

The eating pattern is defined by meal size, duration of a meal and the number of daily meals in beef cattle, and these parameters allow to calculate the daily food intake, daily feeding time and the eating rate (Nielsen, 1999; Devant and Bach, 2017) (**Figure 1**). Consequently, this eating behavior could be affected and/or modulated by a wide range of factors related to the diet (nutrient composition, presentation form), farm facilities and management (feeder space per animal, animals per pen, feed availability) and the animal itself (social behavior, stress, health), being highly important to consider the relationship between them in order to achieve the best performance and efficiency.

On the other hand, complex physiologic and psychological mechanisms are involved in voluntary-intake behavior regulation, by reward and homeostatic systems (Ginane et al., 2015). Homeostatic system is related to the nutritional status and requirements of the animal, and different metabolites regulate hunger and satiety by peripheral signals (nutrients, metabolites, hormones or peptides) that arrives to the brain, regulating food intake (Ginane et al., 2015). The reward system motivates the animal to seek beneficial foods based on previous experiences, and is closely associated with homeostatic system, involving different brain areas and neurochemical pathways (Ginane et al., 2015).

Furthermore, the existence of a communication cross-talk between gastrointestinal tract, microbiota, and the brain has been proposed: the gut-brain axis (Wiley et al., 2017). Thus, through this complex network, inflammation, microbiota, and diet might affect animal behavior (Haagensen et al., 2014) and also the eating pattern.

Certainly, eating behavior may have a significant effect on total voluntary feed intake, hence affecting also feed efficiency, so that mechanisms involved in eating behavior modulation need to be deeply investigated in beef cattle due to its relevance in feed efficiency but also in animal health and welfare.

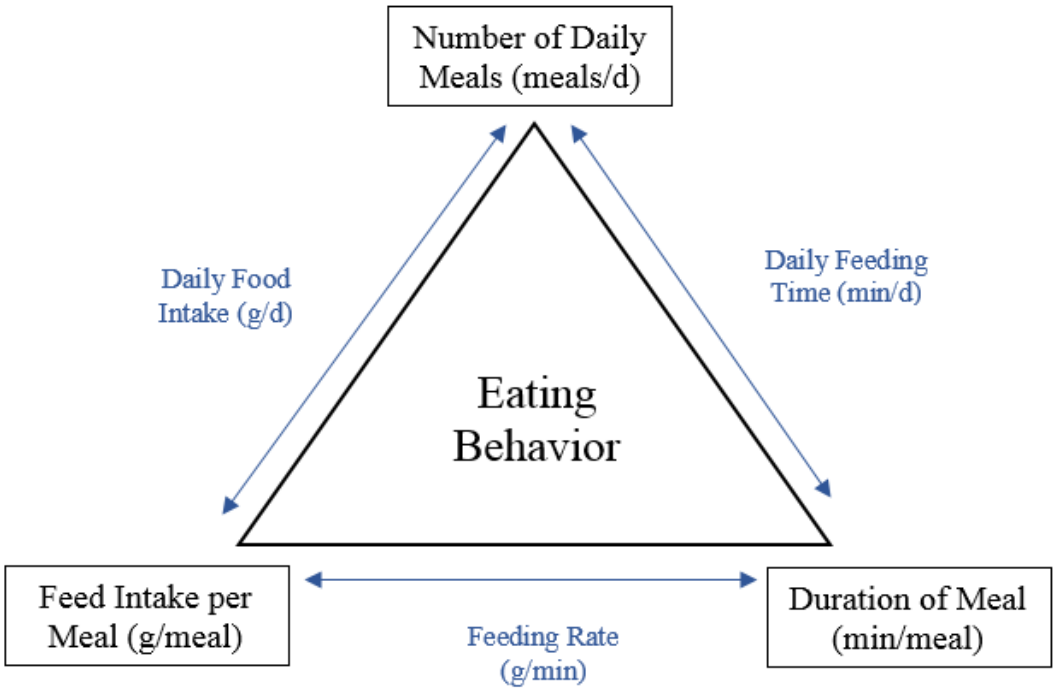

Figure 1. Components of the eating behavior and their interrelations. Adapted from Nielsen (1999).

2. CHEMOSENSORY TRANSDUCTION AND GUT-BRAIN AXIS IN BEEF CATTLE

2.1. Chemosensory transduction and nutrient sensing in beef cattle

The last decades an important number of reports describing the presence of taste and odorant receptors in non-oral and non-olfactory tissues of different vertebrate species has been published. Taste receptors have been found in a wide range of tissues and organs out of the mouth, like gastrointestinal and respiratory tract, brain, skeletal muscle, and testis in different species (Kiuchi et al., 2006; Behrens and Meyerhof, 2011; Beer et al., 2012; Colombo et al., 2012; Foster et al., 2014; Roura and Foster, 2018). The chemosensory transduction is the biological process that allows mammals and organisms to recognize the different chemical stimuli present in the external or internal environment (Zufal and Munger, 2016). These chemical stimuli recognition is basic for the maintenance of life, and plays a key role also in feeding processes, being responsible of identifying palatability, safety, and nutritional value of food (Zufal and Munger, 2016). Thus, taste receptors have the function of detecting and transducing sapid chemicals and,

as a response, releasing transmitters from the cell that would activate the proper response (Breer et al., 2012; Damak, 2016; Roura and Foster, 2018).

The functions of these taste receptors within the digestive tract seem to be involved in important and complex communication pathways between the gastrointestinal system and the brain. Consequently, they might be involved in the homeostatic system acting as a sensors for food-derived components, being key players in the hungry and satiety mechanisms through the release of hormones which could act locally (in a paracrine way) or systematically (by circulatory or lymphatic systems) (Breer et al., 2012; Ginane et al., 2015; Roura and Foster, 2018). Thus, taste receptors would be involved in the modulation of the eating pattern and dietary choices of the animals not only by the taste of food or nutrients in the mouth, but in addition acting as a nutrient sensors thorough the digestive tract. Sweet taste receptors (*T1R2/T1R3*), amino acids taste receptors (as umami taste receptor, *T1R1/T1R3*), bitter taste receptors (*TAS2R*) and various free fatty acid receptors (*ffar*) as *ffar1*, *ffar2, ffar3* and *ffar120*, among others, have been implicated in nutrient sensing thorough the digestive tract in human and different mammal species (Breer et al., 2012; Reimann et al., 2012; Depoortere, 2014; Roura and Fu, 2017). Also salty and sour taste receptors would be included in nutrient sensing mechanisms, thus nutrient sensing receptors repertoire would allow to recognize all tastes (sweet, umami, bitter, soar, salty, and fat), nutrients (carbohydrates, proteins, fat and minerals) and possible toxic molecules (bitter and soar tastes).

In regards to cattle, there is scarce literature focused on nutrient sensing or describing the presence of these taste receptors, either in the digestive tract or in other tissues. Within the digestive tract, the main receptor for sweet taste is the heterodimer formed by the subunits *T1R2* and *T1R3*. In most mammalian species, carbohydrates are one of the most important source of energy, so that sweet receptors main functions described are involved in energy balance, glucose homeostasis, and food intake, acting as an energy sensors (Lee and Owyang, 2017). On the contrary, the main source of energy for ruminant species are VFAs produced by ruminal microorganisms (Siciliano-Jones and Murphy, 1989), so it could be expected to find differences in nutrient sensing and pathways involved in energy balance and food intake between ruminants and non-ruminant species. The particular digestive system of ruminants ferments most dietary starch thanks to ruminal microorganisms, but depending on the diet variable quantities of

starch arrive to the small intestine, where they are hydrolyzed (Mills et al., 1999; Larsen et al., 2009). Moran et al., (2014) demonstrated that *T1R2/T1R3* receptor is expressed in L-endocrine cells of bovine duodenum, and was activated by the presence of glucose. Thus, it might indicate that sweet taste receptor would be acting as energy sensing receptor in the duodenum of cattle, as in other animals does.

VFAs (mainly acetic, propionic and butyric) are the main energy source for ruminant species due to microbial fermentation in the rumen (Siciliano-Jones and Murphy, 1989). Thus, it would be logical that ruminants possessed a nutrient sensing pathway adapted to this particularity, where VFAs would play an important role as cues for energy homeostasis and food intake modulation. The *ffar2* and *ffar3*, previously named *GPCR43* and *GPCR41* respectively, are the targets for short-chain fatty acids (SCFA) (Wang et al., 2009 and 2012; Hudson et al., 2012; Friedrichs, 2015). The mRNA expression of these receptors have been found in many tissues of cattle, especially in the digestive tract, including rumen epithelium, omasum, reticulum, and intestine, mammary gland and adipose tissues (Wang et al., 2009 and 2012; Friedrichs, 2015; Devant et al., 2016; Mielenz, 2017). Actually, it is very interesting that both bovine receptors, *ffar2* and *ffar3*, responded with the lower binding affinity to acetic acid (C2) when compared with human *ffar2* and *ffar3* (Hudson et al., 2012). Thus, it make sense that these receptors exhibit less sensitivity to the predominant SCFA produced in the rumen, possibly resulting in higher tolerance to changes in acetic acid levels. Otherwise, it could explain why propionic acid plays a key role as a regulator of feed intake in ruminants instead of acetic acid, acting as an important hypophagic signal (Bradford and Allen, 2007; Allen et al., 2009 and 2012), as bovine *ffar2* and *ffar3* are more sensitive to propionic acid. Therefore, these affinities of bovine *ffar* could be an adaptation for energy balance regulation, inasmuch as propionic acid increase is related to high-starch diets in cattle and, consequently, to higher energy content in the diet.

Regarding protein or amino acids, there are several nutrient sensing receptors mediating taste of L-amino acids (Behrens and Meyerhof, 2016). The heterodimer formed by the subunits *T1R1* and *T1R3*, named as umami receptor and possessing high affinity for the amino acid L-glutamate, have being widely found in mammals (Zhang et al., 2012; Behrens and Meyerhof, 2016) and also in cattle (Ginane et al., 2011; Zhang et al., 2012).

However, scarce literature is available about these receptors distribution in cattle gastrointestinal tract.

Bitter taste receptors (*TAS2R*) repertoires of mammalian species have been extensively studied (Go, 2005; Dong et al., 2009; Hu and Shi, 2013; Li and Zhang, 2013; Hayakawa et al., 2014), including ruminants (Ferreira et al., 2013 and 2015). Numerous studies in different species are highlighting the implication of *TAS2R* in gastric emptying, satiety and gut motility (Glendinning et al., 2008; Janssen et al., 2011; Depoortere, 2014; Roura and Foster, 2018), and probably cattle *TAS2R* functions within the digestive tract are similar to these described in other species. Although *TAS2R* functions in gastrointestinal tract of cattle have not been profoundly explored, the high tolerance for bitter tastants and the gene repertoire for *TAS2R* of cattle, probably adapted to their diet, could open new insights for studying alternatives to modulate the eating pattern and, consequently, efficiency in this specie.

2.2. Gut-brain axis in beef cattle

Recently, gut-brain-microbiota axis has been proposed as a communication bidirectional network between brain, digestive system and its microbiota in humans and other mammal species, regulating essential functions, such as immunity, digestion, metabolism, hunger and satiety control, and also stress responses (Haagensen et al., 2014; Carabotti et al., 2015; Sarkar et al., 2016; Wiley et al., 2017). Additionally, it has been suggested that inflammation, microbiota, and diet may affect animal behavior (Haagensen et al., 2014) through this gut-brain axis crosstalk. As gut-brain axis is bidirectional, gut microflora signaling mechanisms to central nervous system (CNS) include nervous, endocrine and immune signals, whilst CNS modulates microflora though the autonomic nervous system (Martin et al., 2018).

Basically, four main important routes of communication between brain and microbiota have been described: i) the vagus nerve; ii) gut hormone signaling; iii) the immune system; and iv) different microbial metabolites (Devant et al., 2016; Foster et al., 2017).

Thus, microbiota produces different metabolites involved in the SNC modulation, as short chain fatty acids (which acts over *ffar2* and *ffar3*), secondary bile acids (acts

through G protein-coupled bile acid receptor (*TGR5*)), and tryptophan (as a precursor of serotonin) (Carabotti et al., 2015; Martin et al., 2018). Furthermore, different nutrient sensing receptors are also involve in gut-brain axis, building a very complex network where nutrition, microflora, health and animal behavior are completely interrelated. On the other hand, SNC affects microbiota modulating gut motility, intestinal transit, gut permeability and microbiota gene expression trough the secretion of different hormones (Martin et al., 2018).

One of the mechanisms of the gut-brain axis that could play an important role in animal welfare, affecting health but also animal behavior, would be inflammation (Haagensen et al., 2014; Devant et al., 2016; Wiley et al., 2017). Inflammation, among other mechanisms, is associated with a decrease in serum concentrations of serotonin, a monoamine neurotransmitter related with well-being, mood modulation and a reduction in aggressive and sexual behaviors (Evans et al., 2013; Haagensen et al., 2014; Devant et al., 2016).

Although scarce literature is available in beef cattle, the gene expression of different receptors involved in crosstalk mechanisms of the gut-brain axis (*ffar3*, *ppyr1*, *adra2c*, *occluding* and *TNFα*) have been found in the rumen of bulls, suggesting that some of these gut-brain crosstalk mechanisms could take place there (Devant et al., 2016). Due to the importance of these mechanisms on animal feeding behavior, welfare and health and to the particularities of digestive system in beef cattle, further research is needed to deeply understand them and being able of modulate them as well.

3. FEED ADDITIVES IN BEEF CATTLE

Accordingly to the definition of the European Regulation (EC) No 1831/2003, ‘feed additives’ means substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, the quality of feed and the quality of food from animal origin, or to improve the animals’ performance and welfare, or reducing environmental consequences of animal production.

This European Regulation also classifies feed additives in different categories, based on their functionality and properties, and one additive could be allocated in one or more of these categories:

- Technological additives: any substance added to feed for a technological purpose (e.g. preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, and silage additives).
- Sensory additives: any substance, the addition of which to feed improves or changes the organoleptic properties of the feed, or the visual characteristics of the food derived from animals (e.g. flavorings, colorants).
- Nutritional additives: substances performing a nutritional function (e.g. vitamins, minerals, amino acids, trace elements).
- Zootechnical additives: any additive used to improve the performance of animals, health or used to reduce the environmental impact of animal production.
- Coccidiostats and histomonostats: feed additives intended to kill or inhibit protozoa microorganisms.

This classification could be adapted to technological progress or scientific development. In fact, this legislation is actually being modified, and probably a new group of Zootechnical additives will be included, as physiological condition stabilizers, closely related with animal welfare.

Focusing on beef cattle fattened under intensive conditions, we will briefly describe the functionality and possible applications of feed additives described in the literature to:

- Reduce ruminal methanogenesis and improve efficiency.
- Control of rumen acidosis in beef cattle.
- Improve beef cattle health.

3.1. Feed additives to reduce ruminal methanogenesis

As previously explained, ruminant livestock substantially contributes to GHG emissions due to methane production during ruminal fermentation. Normally, beef cattle fattened under intensive conditions are fed high-concentrate diets, producing less

methane than cattle produced in extensive systems (IPCC, 1997; Moss et al., 2000; Steinfeld et al., 2006). Moreover, reducing methane production is not only an environmental concern, being also nutritionally important due to energy losses and feed efficiency impairment. Therefore, reducing methane emission is also related to an improvement in efficiency and productivity (Steinfeld et al., 2006; Giuburunca et al., 2014).

As methane is produced during ruminal fermentation, obviously we can reduce methane emission by different ways, 1) reducing feed intake (if there is less ruminal content, ruminal fermentation decrease and also methane production and emission), 2) modulating ruminal fermentation (acting directly over microorganisms involved in methane production during ruminal fermentation or, indirectly, reducing the substrates they use to produce methane) or 3) improving animal health (enhancing animal productivity by reducing pathology) reducing improductive days (Knapp et al., 2014).

Different plant secondary metabolites, defined as plant chemical compounds not involved in plant growth or reproduction (Patra and Saxena, 2010), have been studied and proposed for reducing ruminal methane production. Within this group, essential oils, tannins, saponins, and flavonoids have been proposed as potential alternatives to reduce ruminal methane production and emission (Patra and Saxena, 2010; Cobellis et al., 2016a and 2016b; Rira et al., 2019). Also the use of 3-nitrooxypropanol (3-NOP), an enzymatic inhibitor, has clearly showed positive effects in reducing methane production in beef cattle fed high-concentrate diets (Romero-Perez et al., 2014; Kim et al., 2019).

3.1.1. Essential oils

Essential oils are secondary metabolites produced by plants in complex mixtures with heterogenic chemical composition, and possess different antimicrobial properties (Cobellis et al., 2016b). Thus, depending on the composition of the essential oil the effect exhibited over methanogenesis will be different (Patra and Saxena, 2010; Cobellis et al., 2016a). Different essential oils and combinations have showed promising effects over ruminal microorganisms, reducing both archaea and protozoa and, consequently methane production. But also some negative effects over nutrient digestibility (protein and starch), VFAs production and intake have been observed in beef cattle (Patra and Saxena, 2010;

Cobellis et al., 2016a and 2016b), probably due to their antimicrobial properties along with some problems related to palatability (Calsamiglia et al., 2007).

Consequently, although essential oils are a very promising molecules to reduce methane production, extensive research is needed to properly determine active compounds, synergisms and antagonisms between them, on-farm doses and mode of action (Cobellis et al, 2016b). Additionally, manufacturing feed additives based on essential oils to be used on-farm need standardized and optimized processes to guarantee a stable composition and quality of the products (Stevanovic et al., 2018).

3.1.2. Tannins.

Tannins are polyphenolic compounds naturally found in so many species of plants, and normally classified in hydrolysable or condensed tannins, depending on their structure (Patra and Saxena, 2010; Rira et al., 2019). The anti-methanogenic effects of tannins are been largely demonstrated, although this effect has been also related to lower digestibility of feed due to the formation of complexes with proteins along with a negative impact over ruminal populations (Patra and Saxena, 2010; Piñeiro-Vázquez et al., 2015; Rira et al, 2019).

Different tannins from different plant sources have leaded to a reduction in methane production in different *in vivo* and *in vitro* studies, and probably by exerting a direct inhibitory effect over methanogens but also by an indirect effect over ruminal protozoa (Patra and Saxena, 2010). As in the case of essential oils, different tannins from different plant sources trigger contrary responses, so standardized processes and the study of possible interactions with other substances naturally present in feedstuffs are required for practical applications.

3.1.3. Saponins

Saponins are glycoside molecules with high molecular weight naturally found in a wide variety of plants and possess different biological properties depending on their chemical structure (Patra and Saxena, 2010). In this case, the research has been focus on saponins' effects on rumen ciliate protozoa and, as with different plant secondary metabolites occurs, methane reduction in the rumen depends on the level of inclusion and

also the source of saponins (Jayanegara et al., 2014). Saponins reduce protozoa concentration, but also cellulolytic and anaerobic fungi in the rumen, leading to a decrease in hydrogen availability for methanogen populations, so methane production is consequently reduced (Jayanegara et al., 2014). Moreover, in the metanalysis carried out by Jayanegara et al. (2014), different levels of saponins extracted from Quillaja, Tea and Yucca did not negatively affect digestibility and total VFAs production on *in vitro* rumen fermentation systems.

3.1.4. Flavonoids

Flavonoids are a large group of secondary plant polyphenols that possess important biological properties as antioxidant and chelating molecules, but also with interesting anti-microbial effects (Patra and Saxena, 2010). Recent studies have reported a significant reduction in methane production by depressing methanogenic archaea communities in different *in vitro* studies performed with ruminal liquid from steers fed high concentrate diets (Seradj et al., 2014) and animals fed forage-rich diets (Wang et al., 2013).

3.1.5. 3-Nitrooxypropanol

The molecule 3-nitrooxypropanol is a synthetic enzymatic inhibitor that has demonstrated to be an effective feed additive reducing enteric methane production in ruminants, including beef cattle (Romero-Perez et al., 2014; Kim et al., 2019). The mechanism of action consists in targeting the enzyme methyl-coenzyme M reductase in rumen archaea, inhibiting the last step for methane production by this population, being very specific and effective at low doses on *in vivo* trials (Duin et al., 2016).

3.2. Feed additives to control rumen acidosis in beef cattle

Acidosis is considered a common metabolic disorder with ruminal origin in beef cattle fed high-concentrate diets. As a metabolic disorder of the gastrointestinal tract, acidosis affects performance and health, resulting also in animal welfare impairment. Basically, acidosis could be defined as a reduction in ruminal pH, affecting ruminal microorganism populations and, as a consequence, ruminal fermentation, intake and

performance. Thus, different feed additives are commonly used in beef cattle fed high-concentrate diets to control and reduce ruminal acidosis effects in intensive production systems: buffers (as sodium bicarbonate) or alkalizers (as magnesium oxide), ionophores (as Monensin), probiotics (as yeasts), and organic acids (as malic, fumaric, and aspartic acid) (Bach et al., 2007; Calsamiglia et al., 2012; González et al., 2012; Hernández et al., 2014). In our production system, dairy calves, where animals are raised from the weaning phase with concentrate and straw both ad libitum with separate feeders the risk of suffering rumen acidosis is low (Devant et al., 2016) except to situations where management is poor: no straw at the feeders, high pen density that reduces the options to access to the feeders or periods where no feed is in the feeders due to delivery problems. Therefore, in the praxis, in the commercial feed formulas, these feed additives are used to reduce or prevent the risk of suffering rumen acidosis.

3.2.1. Buffers and alkalizers

Sodium bicarbonate is widely used in beef cattle fed high-concentrate diets for its ruminal buffering capacity (Calsamiglia et al., 2012). Basically, bicarbonate is the natural buffer present in the saliva, so addition of sodium bicarbonate exerts a direct effect on ruminal pH, increasing buffer capacity of ruminal fluid, although some contradictions around its dosage and response still exists (Calsamiglia et al., 2012; González et al., 2012).

Magnesium oxide is the most commonly used alkalizer, exerting a direct effect increasing ruminal pH, but palatability problems should be considered (Calsamiglia et al., 2012; Hernández et al., 2014). This product is mainly used in dairy cattle, due to a positive effect on content of fat in milk observed when magnesium oxide is supplemented, more than its impact on ruminal pH (Calsamiglia et al., 2012).

However, the positive effects of buffers and alkalizers in the prevention of ruminal acidosis seem to be limited (Hernández et al., 2014) as they work, buffers within a range of pH between 7 and 6 which is not critical and alkalizers work once the pH is low and most rumen acidosis consequences of rumen acidosis are already taking place.

3.2.2. Ionophores

Monensin is an antibiotic and is the most frequent ionophore used as feed additive in beef cattle worldwide, although it was banned in Europe in 2006. Recently, due to the relevance and growing concern about bacterial resistance to the antibiotics, and its impact on human health, the prohibition of the use of antibiotics as a growth promoters in animal feed is being spread out to other countries around the world.

Basically, monensin modifies ruminal microorganism populations, increasing propionate and reducing acetate, so improving efficiency (González et al., 2012). Furthermore, this increase in propionate results in an eating pattern modulation, reducing meal sizes and increasing the frequency of meals, so promoting lesser ruminal pH fluctuations and reducing ruminal acidosis (González et al., 2012).

3.2.3. Probiotics

Regarding probiotics in beef cattle, although some bacteria alternatives (as *Megasphaera elsdenii*) have been studied and proposed, yeasts and especially different strains of *Saccharomyces cerevisiae*, have showed positive effects on ruminal pH. Yeasts would modulate ruminal fermentation increasing lactate consumption by different ruminal bacteria and increasing ruminal pH as a result (Calsamiglia et al., 2012; Hernández et al., 2014). Also a modulation of the eating pattern in dairy cattle has been described when supplementing with a strain of *Saccharomyces cerevisiae*, increasing meal frequency of the animals (Bach et al., 2007).

3.2.4. Organic acids

Organic acids are considered safe technological feed additives (in the EU register). Focusing on beef cattle, malic, fumaric and aspartic acid basically would stimulate bacteria utilizing lactic acid, as *Selenomonas ruminantium* (Calsamiglia et al., 2012). Thus, the use of these organic acids would increase ruminal pH, also propionate and total VFA, and the higher pH would promote the growth of fibrolytic bacteria, improving ruminal environment (Calsamiglia et al., 2012; Hernández et al., 2014).

In summary, to regulate rumen acidosis most of the previous mentioned additives affect the rumen flora (or at least these are the ones that have been described). Few research has been conducted in how these additives directly or indirectly can modify the gut (rumen) metabolism: maybe they modify inflammation, or maybe they modify the eating pattern through altering the signaling of receptors involved in feed intake. As the effect of those additives seems to differ depending on diet one could hypothesize that probably those mechanisms are linked to the modification of the rumen flora.

3.3. Feed additives to improve beef cattle health

After prohibition of antibiotics as growth promoters in livestock production, an important research to find and develop additives that could exert beneficial effects on the immune system and improve the health of the animals has been performed. Obviously, due to the close relation between health, productivity and animal welfare, to find alternatives to the use of antibiotics is an outstanding target in our intensive animal production systems.

Focusing in beef cattle, probably ruminal acidosis and bovine respiratory disease (BRD), are both the most important health challenges in fattening farms. On the one hand, ruminal acidosis is often related to different health problems by rumenitis and parakeratosis of the ruminal epithelium, that would predispose the animal to other different diseases such as laminitis, endocarditis, bloat, liver abscesses, and BRD (Chaucheyras-Durand and Durand, 2010; Hernández et al., 2014). On the other hand, BRD supposes to beef cattle industry an important detriment due to the losses in performance, health and animal welfare impairment, being the major cause of clinical disease and death in beef cattle reared under intensive production systems (Edwards, 2010; Hay et al., 2016). Pathogens involved in BRD are commonly found in the upper respiratory tract of the animal, but this is a multifactorial pathology that also involves host susceptibility (stress and immunological status), and animal management (Edwards, 2010; Hay et al., 2016).

Therefore, controlling and reducing ruminal acidosis would lead to a health improvement, reducing the predisposition to suffer other pathologies, and would improve immune status of the animal as well. Additionally, ameliorating the immune status of beef cattle would help to reduce the development of BRD during the fattening period.

Consequently, the feed additives previously explained to control and reduce ruminal acidosis would play also an important role by improving health and immune status in cattle.

Immunomodulators are different substances capable to positively affect immune response of the animal to prevent or control disease (Blecha, 2001). Thus, the most important objective of these immunomodulatory feed additives would be acting on inflammation and immune function (IRTA, 2015), consequently that would be a wide and diverse group of additives and molecules, such as probiotics, prebiotics, plant extracts and phytochemicals, some vitamins and nutrients (Blecha, 2001; Chaucheyras-Durand and Durand, 2010; IRTA, 2015).

4. FEED ADDITIVES AND ANIMAL BEHAVIOR

Dinan et al. (2013) defined a "psychobiotic as a live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness". Thus, based on this definition, psychobiotics are in fact probiotics that produces and releases neuroactive substances which act on the gut-brain axis (Dinan et al., 2013). After further research, Sarkar et al. (2016) expanded this definition of psychobiotic including also prebiotics which specifically promote beneficial gut bacteria, positively affecting mental health with psychophysiological effects.

Thereby, these psychobiotics act through the gut-brain axis (see **2.2. Gut-brain axis in beef cattle)** producing neuroactive substances, such as serotonin and gamma-aminobutyric acid, which have the ability of affecting cognition, memory, learning and behavior, also modulating mood and anxiety (Dinan et al., 2013; Sarkar et al., 2016; Bermúdez-Humarán et al., 2019).

Obviously, this is a truly incipient research basically based on rodent models, but it would be easy to think that these mechanisms and effects should exist in all mammal species probably adapted to microbiota and diet. Thus, it could be expected that probiotics, prebiotics and different feed additives commonly used in livestock feeding systems would be also exerting these effects though the gut-brain axis cross-talk mechanisms. Actually, considering the evident importance of ruminal microbiota, which includes a wide range and density of bacteria, protozoa, archaea and fungi populations, it

could be hypothesized that modulating this microbiota we might also modulate animal behavior.

This hypothesis would open an interesting area of research in cattle, but also in other domestic animals, to study new alternatives as probiotics or prebiotics with the capacity of positively modulate and improve animal behavior, health and welfare, so developing psychobiotics for livestock. Thus, previously mentioned, reducing oral non-nutritive sexual, and aggressive behaviors in intact bulls for fattening is needed to improve animal wellbeing among others benefits (animal handling, meat quality, etc…).

5. PREVIOUS RESEARCH OF CITRUS FLAVONOIDS IN BEEF CATTLE

Flavonoids are polyphenols with a great number of biological properties, such as anti-inflammatory, antioxidant, and antimicrobial activity (Harborne and Williams, 2000). These polyphenols have been deeply studied due to their positive effects on human health (Heim et al., 2002). A high range of flavonoids have been found in vegetables, being classified based on their chemical structure, which also affects their biological properties (Harborne and Williams, 2000; Heim et al., 2002). Citrus fruits are considered as one the major source of flavonoids in human diet.

Previous research have been performed with citrus flavonoids, *in vivo* and *in vitro*, to study the possible positive effects in beef cattle. Balcells et al. (2012) found beneficial effects of supplementing a citrus flavonoid commercial product in heifers fed high-concentrate diets. Citrus flavonoids supplementation increased lactic acid consuming bacteria (*Megasphaera elsdenii*) and, consequently, improve ruminal pH. Furthermore, also an increase in the molar proportion of propionic acid was obtained, reducing the actetate:propionate ratio in heifers supplemented with citrus flavonoids.

On the other hand, *in vitro* studies (Seradj et al., 2014) performed with the same citrus flavonoid commercial product showed promising effects reducing methane production by depressing methanogenic archaea communities.

Consequently, all these positive effects could lead to an improvement in productivity, health and efficiency in beef cattle fed high-concentrate diets.

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CHAPTER II

Objectives

1. OBJECTIVES

Nowadays, it exists an outstanding need of improving animal health, welfare and productivity in livestock reared under intensive conditions. Additionally, this challenge is even greater when considering the imperative need of reducing the use of antibiotics in our animal production systems. Consequently, developing natural and effective alternatives that help us to achieve these goals is almost mandatory.

Citrus flavonoids are polyphenols that possess interesting anti-inflammatory, antioxidant, and antimicrobial properties. Previous research carried out with citrus flavonoids in fattening beef have showed positive effects in some rumen parameters:

- ✓ Greater rumen pH, increasing the concentration of bacteria consuming lactic acid as *Megasphaera elsdenii*.
- ✓ An *in vitro* reduction of methane production depressing methanogenic archaea communities.
- ✓ Ruminal VFA modification, increasing molar proportion of propionic acid.

Consequently, improving ruminal pH could improve ruminal health, reducing the incidence of ruminal acidosis. Furthermore, increasing molar proportion of propionic acid and reducing methane production, both could positively affect productivity and efficiency of beef cattle. Additionally, propionic acid acts as an important regulator of feed intake in cattle fed high-concentrate diets, so citrus flavonoids could affect eating pattern when high-concentrate diets are fed, however the effect of citrus flavonoids on eating pattern has not been previously evaluated.

Otherwise, recently the existence of a communication network between gastrointestinal tract, microbiota, and the central nervous system, the gut-brain axis, has been proposed in mammals. Thus, inflammation, microbiota, and diet might be affecting animal behavior (Haagensen et al., 2014). Therefore, the effect of supplementing citrus flavonoid could affect animal behavior and wellbeing and this effect could be modified by diet.

Having these hypothesis in mind the main objective of this thesis was to study the possible benefits of supplementing citrus flavonoids in bulls fattened under intensive

conditions and fed high-concentrate diets on performance and productivity, eating and animal behavior.

To achieve this target, different specific objectives were defined:

1. To study the eating pattern behavior of bulls fed high-concentrate diets when citrus flavonoids were supplemented, evaluating concentrate intake, growth rate, and concentrate efficiency.

2. To evaluate the possible effects of citrus flavonoids supplementation on animal behavior under farm commercial conditions.

3. To investigate the effects of supplementing citrus flavonoids on rumen health parameters, ruminal VFA content and composition, and ruminal pH at slaughterhouse.

4. To evaluate the possible interaction of citrus flavonoids supplementation with the concentrate presentation (pellet vs. meal) and composition (fat level) in bulls fattened under commercial conditions.

5. To analyze the possible effects of citrus flavonoids on nutrient sensing receptors involved in hunger and satiety regulation, inflammation, and behavior that have been related with the gut-brain axis.

To accomplish these specific objectives, four studies were performed:

1. Study 1: designed to evaluate the effects of citrus flavonoids supplementation on eating pattern, concentrate consumption, growth rate, feed efficiency, rumen wall health, carcass characteristics, and animal behavior in Holstein bulls fed high-concentrate diets in pellet form, using a single-space feeder.

2. Study 2: designed to evaluate the effects of citrus flavonoids supplementation in bulls fed high-concentrate diets in pellet form on performance (concentrate consumption, growth and concentrate efficiency), carcass characteristics, rumen wall health and animal behavior in commercial conditions (multi-space feeders). Additionally, the expression of genes involved in the gut-brain axis crosstalk, such as nutrient sensing receptors, some neurotransmitters receptors and different inflammation regulators in rumen epithelium were studied.

3. Study 3: designed to evaluate the effects of citrus flavonoid extract supplementation on concentrate consumption, growth rate, feed conversion ratio, macroscopic rumen wall health, carcass characteristics, and eating and animal behavior of Holstein bulls fed high-concentrate diets in meal form fattened under commercial conditions (multi-space feeder). Furthermore, the expression of genes in the rumen epithelium involved in gut-brain crosstalk mechanisms such as taste receptors and inflammation regulators was analyzed.

4. Study 4: designed to evaluate the effects of citrus flavonoid supplementation on concentrate consumption, growth rate, concentrate efficiency, macroscopic rumen wall health, carcass characteristics, and animal behavior in Holstein bulls fed high-fat concentrate diets at the finishing phase in meal form under commercial conditions. Additionally, the expression of some genes involved in gut-brain crosstalk mechanisms in the rumen and duodenum epithelium, such as nutrient sensing receptors and inflammation regulators, was evaluated.

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CHAPTER III

Effects of flavonoids extracted from *Citrus aurantium* on performance, eating and animal behavior, rumen health, and carcass quality in Holstein bulls fed high-concentrate diets.

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ABSTRACT

The effects of flavonoids extracted from Citrus aurantium (Bioflavex® CA) on eating pattern, performance, carcass quality, and rumen wall health of Holstein bulls fed on a single feeder were studied. One hundred ninety-eight bulls (195.3 ± 19.6 kg of body weight and 149 ± 6.8 d of age) were used in a complete block randomized design. Groups of animals with the same mean and coefficient of variation of body weight (replicates) were randomly allocated in 1 of 6 pens (20 animals per pen), and each pen was assigned to a Control (C) diet or to a diet supplemented with flavonoids (Bioflavex® CA, Interquim S.L., Spain) (BF, 0.4 kg per ton of concentrate of Bioflavex® CA) in two consecutive fattening cycles. Concentrate intake was recorded daily, and BW fortnightly. Animal behavior was monitored by visual scan procedure every fourteen days. Animals were slaughtered after 168 d of study, hot carcass weight and carcass quality were recorded, and internal rumen wall was examined. Concentrate intake was higher ($P < 0.05$) in C than in BF bulls; however, ADG and concentrate efficiency were not affected by treatments. The final BW tended ($P = 0.06$) to be higher in C than in BF bulls, but this difference disappeared for carcass weight. In the finishing phase, the proportion of meal size values above 750 g was higher ($P < 0.05$) in C compared with BF bulls. Throughout the study, BF bulls spent more time eating straw ($P < 0.01$) and concentrate ($P < 0.05$), and exhibited more displacements ($P < 0.05$) than C bulls, whilst C group performed more ($P < 0.05$) oral behaviors. Bulls of C treatment exhibited more ($P < 0.01$) fighting than BF group throughout the study, and butting tended to be higher ($P < 0.09$) for the growing phase, and higher ($P < 0.001$) for the finishing phase in this group compared with C bulls. During the finishing phase, sexual behaviors such as flehmen and complete mounts were higher ($P < 0.01$ and $P < 0.05$, respectively) in C bulls as well, and C bulls tended ($P = 0.10$) to perform more attempted mounts compared with BF bulls. In the slaughterhouse, color of rumen wall tended ($P = 0.06$) to be lighter for BF compared with C bulls, and presence of baldness areas in the rumen was lesser ($P = 0.01$) in BF animals. In conclusion, when bulls were supplemented with citrus flavonoids, feed intake was reduced. Citrus flavonoid supplementation increased time eating straw, reduced agonistic behaviors throughout the study and sexual interactions during the finishing phase, potentially improving animal welfare. Rumen wall parameters analyzed were indicative of a better rumen health in BF than in C bulls, which may be due to the reduction of large meal sizes.

Keywords: behavior, bulls, flavonoids, meal size, performance, rumen health.

1. Introduction

Flavonoids are widely distributed in the plant kingdom, i.e. in fruits, seeds, vegetables, tea, and wine. Some of these compounds have anti-inflammatory, antioxidant, and antimicrobial properties (Harborne and Williams, 2000). Due to their interesting capabilities, flavonoids from different sources are being studied for different applications in animal production. Bioflavex® CA (Interquim, S.A., Spain) is an extract from bitter orange (*Citrus aurantium*) whose major flavonoid is naringin. Naringin is a glycosylated flavanone classified into the neohesperidoside type, with a neohesperidose (rhamnosyl-α-1,2 glucose) attached to its basic structure as a flavanone (Tripoli et al., 2007). Other extracts containing naringin have been shown to have beneficial effects in regulating rumen pH in fattening beef (Balcells et al., 2012), as well as reducing *in vitro* methane production from steers fed high concentrate diets (Seradj et al., 2014). Properties of naringin may affect rumen microflora, increasing the concentration of bacteria which consume lactic acid such as *Megasphaera elsdenii* (Balcells et al., 2012; Seradj et al., 2014) resulting in a higher ruminal pH (Balcells et al., 2012), and a depression of methanogenic archaea communities (Seradj et al., 2014). Rumen volatile fatty acids (VFA) composition has been modified as well, increasing molar proportion of propionic acid (Balcells et al., 2012). As propionic acid is an important regulator of feed intake in ruminants fed high-starch diets, affecting both satiety and hunger (Oba et al., 2002), the supplementation of flavonoids could affect eating pattern of bulls fed high-concentrate diets. Moreover, this supplementation could reduce methane production, and together with the reduced ruminal pH fluctuations (Lam, 2016) could increase efficiency of nutrient utilization in steers.

Otherwise, a communication network was described between gastrointestinal system, microbiota, and the central nervous system (Wiley et al., 2017), and thus inflammation, microbiota, and diet may affect animal behavior (Haagensen et al., 2014). As flavonoids act as potent anti-oxidant and anti-inflammatory molecules (Harborne et al., 2000; Heim et al., 2002; Tripoli et al., 2007), they are able to modify VFA composition in ruminal fluid (Seradj et al., 2014), and may alter rumen microflora (Balcells et al., 2012; Seradj et al., 2014); so they could improve animal behavior through the gut-brain axis crosstalk.

The hypothetical benefits of supplementing citrus flavonoids on eating pattern and animal behavior in fattening bulls have not been previously addressed. The present study was designed to evaluate the effects of citrus flavonoids supplementation on eating pattern, concentrate consumption, growth rate, feed efficiency, rumen wall heatlh, carcass characteristics, and animal behavior in Holstein bulls fed high-concentrate diets.

2. Materials and methods

2.1. Animals, Feeding, Housing, and Experimental Design

The study was conducted in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado, 2013). Animals were fattened under commercial conditions in a farm (Agropecuaria Montgai SL, Montgai, Lleida). One hundred ninety-eight Holstein bulls (195.3 ± 19.6 kg of body weight (BW) and 149 ± 6.8 d of age) in two consecutive fattening cycles (99 animals each cycle) were used.

Animals were randomly allocated in one of six covered pens (12 m long x 6 m wide) that were deep-bedded with straw and equipped with a computerized concentrate single-space feeder (0.50 m long x 0.26 m wide x 0.15 m depth) with 10 kg of concentrate capacity as described elsewhere (Verdú et al, 2015), with lateral protections (1.40 m long x 0.80 m high) forming a chute, which width could be adapted from 42 to 72 cm, depending on the animal size and age (Verdú et al., 2015). This computerized feeding system was calibrated weekly. When each animal visited the feeder, it was identified, the computer recorded the initial and final concentrate's weight, with its initial and final time. Animals were adapted during 3 wk by widening the chute to facilitate feeder access (adaptation period). During the study, the width of the chute has been adapted to the animal size to allow them to eat easily.

Pens were also equipped with a water bowl and a separated straw feeder (3.00 m long x 1.12 m wide x 0.65 m depth; 7 feeding spaces) where straw was offered ad libitum.

2.2. Feed Intake and Performance

Animals were fed a commercial concentrate in pellet form, formulated to accomplish the nutritional requirements of this type of animals (NRC, 2001). The first 112 d of the study, animals were fed a grower concentrate, between 112 d to the end of the study, animals were fed a finisher concentrate. Ingredients and nutrients of the concentrate formulas are presented in **Table 1**. During the study, animals had ad libitum access to wheat straw (3.5 % CP, 1.6 % ether extract, 70.9 % NDF, and 6.1 % ash; DM basis) and fresh water.

Table 1. Ingredients and nutrient composition of the concentrates.

Item	Growing[1]	Finishing[2]
Ingredients, g/ kg		
Corn grain meal	399.8	449.6
Gluten feed	230.0	213.1
Barley grain meal	138.2	108.7
Wheat	110.2	110.1
Beet pulp	49.0	49.9
Palm oil	23.8	27.5
Soybean meal	16.0	16.0
Calcium carbonate	16.0	12.9
Urea	8.0	4.2
Bicarbonate	4.0	4.0
Vitamin premix	3.0	2.0
Salt	2.0	2.0
Nutrients		
ME, Mcal/ kg DM	3.18	3.24
CP, g/ kg DM	152	136
Ether extract, g/ kg DM	53	58
Ash, g/ kg DM	61	55
NFD, g/ kg DM	185	178
NFC, g/ kg DM	548	572

[1] from 0 to 112 days of the study.
[2] from 113 days to the end of the study.

The study was a complete block randomized design. Groups of animals with the same mean and coefficient of variation of body weight (replicates) were randomly allocated in 1 of 6 pens (20 animals per pen), and each pen was assigned to one of the two treatments (3 pens per treatment), either control (C) or supplemented (BF) with 0.04 % of bitter orange extract (*Citrus aurantium*) of the whole fruit rich in naringin, >20% (Bioflavex® CA, Interquim, S.A., Barcelona, Spain) in two consecutive fattening cycles. The dose of 0.04% was based on preliminary field and research studies (Balcells et al., 2012).

Animals were weighed individually every 14 d throughout the study in 12 experimental periods of 14 d, during the 8 first periods (from 1 d to 112 d) the animals consumed the growing concentrate and during the last 4 periods (from 113 d to 168 d) and during the days before slaughter animals consumed the finishing concentrate (see Table 1). After 168 d of study animals were slaughtered within the following 3 weeks, each time one pen from C and one from BF bulls were slaughtered. Transport distance to the slaughterhouse (Escorxador del Grup Alimentari Guissona, Guissona, Spain) was approximately 35 km. The time waiting before slaughter was less than 6 h. Animals were weighed before loading. They were slaughtered by commercial practices and following the EU Regulation 1099/2009 on the protection of animals at the time of killing or slaughtering .Hot carcass weight (HCW) of every animal were recorded.

2.3. Chemical Analyses

During the study, samples of concentrate were collected at d 0, 42, 84, 126, and 168 d. and analyzed for DM (24 h at 103ºC), ash (4 h at 550ºC), CP by the Kjeldahl method (method 981.10; AOAC, 1995), ADF and NDF according to Van Soest et al. (1991) using sodium sulfite and alpha-amylase, and EE by Soxhlet with a previous acid hydrolysis (method 920.39; AOAC, 1995).

Naringin was determined for every sample as a Biofalvex® CA marker for BF group, and was used as a quality control analysis to guarantee the correct addition of the product into the feed by Laboratory of Interquim S.A. Internal method for naringin quantification using HLPC developed by Interquim S.A. was used and analyzed as described herein. To analyze naringin all concentrate samples were milled. Five grams

were weighed and 50 mililiters of dimethyl sulfoxide were added and agitated for 15 min, and was filtered and placed in a vial. The pattern was prepared, 30 mg of naringin were mixed with dimethyl sulfoxide until 100 ml were achieved. Drying losses were taken into account for calculations. Nova-Pak C18 columns were used as stationary phase for the chromatography, silica-based, reversed-phase C18 columns that are based on 4 µm particle technology (Waters Cromatografia SA, Cerdanyola del Vallés, Barcelona). The column was maintained at 40ºC, acidified water with methanol R (70:30) v/v was used as mobile phase, with a flow rate of 1.0 mL/min. 10 µL were injected, and detection was done by UV at 284 nm. The chromatography duration was around 35 min.

2.4. Animal Behavior

A visual scan procedure at days 16, 31, 44, 59, 72, 87, 100, 114, 128, 142, 157, and 168 of the study was performed to study the general activity (standing, lying, eating, drinking, and ruminating) and social behavior (nonagonistic, agonistic, and sexual interactions) of the animals in every pen. Social behavior activities recorded are described in **Table 2**. The visual observation was made for 2 pens at the same time from 8:00 to 10:00 h, as described by Mach et al. (2008), Rotger et al. (2006), Robles et al. (2007), and Martí et al. (2010). General activities were scored using 3 scan samplings of 10 s at 5 min intervals, and social behavior was scored during three continuous sampling periods of 5 min. This scanning procedure of 15 min was repeated twice consecutively in each pen, starting randomly in a different pen every scanning day. This method describes a behavior exhibited by an animal at a fixed time interval (Colgan, 1978).

2.5. Carcass Quality

After slaughtering, HCW was registered for every animal. Dressing percentage was calculated by dividing HCW by BW recorded before slaughtering. Following the (S)EUROP categories described by the EU Regulation No. 1208/81 and 1026/91, conformation of carcasses was classified, where "E" corresponded to an excellent conformation, "U" to very good conformation, "R" to good conformation, "O" to fair conformation, and "P" to a poor conformation. The fat cover was classified according the EU Regulation No. 1208/81, which utilizes a classification system by numbers, 1.2.3.4.5,

where 5 (very high) describes an entire carcass covered with fat and heavy fat deposits in the thoracic cavity, and 1 (low) describes low to none fat cover.

Table 2. Description of the social behavioral categories recorded.

Interactions	Item	Definition
Nonagonistic interactions	Self-grooming	No stereotyped licking of its own body, scratching with a back limb or against the fixtures.
	Social behavior	Licking, nosing with the muzzle or horning a neighboring bull.
	Oral non-nutritive behavior	Licking or biting fixtures with non-nutritive finality.
Agonistic interactions	Fighting	When bulls pushed vigorously head against head.
	Butting	When one bull push vigorously its head against any part of another bull's body.
	Displacement	When one bull jostle itself between 2 other bulls or between a bull and any equipment.
	Chasing	When a bull follow fast or run behind another bull.
	Chasing-up	When a bull push a resting animal and make him to stand up.
Sexual interactions	Flehmen	Upper lip reversed.
	Attempted mounts	Head on the back of another animal.
	Completed mounts	Forelimbs on the back of another animal.
Stereotypies	Oral Stereotypes	Tongue rolling, stereotyped licking or biting any equipment.

2.6. Rumen and Liver Macroscopic Evaluation

Rumen and liver of every animal were macroscopically evaluated at the slaughterhouse. Rumens were classified depending on the color by a visual evaluation, from 1 to 5, being "5" a black colored rumen and "1" a white colored rumen (González et al., 2001). They were also divided into areas according to Lesmeister et al. (2004) to examine the presence of ulcers, baldness regions, and clumped papillae (Nocek et al., 1984). Liver abscesses were classified according to Brown et al. (1975).

2.7. Calculations and Statistical Analyses

Pen was considered the experimental unit and animals within pen were considered observational units for all statistical analyses**.** Two pens (one of the C group and one of

the BF group) belonged to the first fattening cycle were removed due to technical problems with the antenna of the single-space feeder, and all data of these animals were deleted from the databases.

Meal criteria for each animal and period was calculated as described by Bach et al. (2006). Thus, visits at the single-space feeder were separated into meals, and eating pattern parameters (meal frequency, meal duration, inter-meal duration, and meal size) were calculated. To calculate performance, eating behavior and concentrate consumption, all individual data registered were averaged by the experimental period (14 d period). The percentage of mean meal size above 750 g was estimated, the criterion of 750 g was chosen based on the distribution of the meal size using all data (all animals and all periods), 750 g was the average meal size. In addition, Nielsen (1999) in their review observed a negative relationship between meal size and feeder visits, and above 750 g of mean meal size this relationship is not linear, in consequence above 750 g of meal size the number of visits to the feeder are reduced limiting total daily feed intake. Concentrate efficiency data were transformed into log to achieve a normal distribution. The means presented in the tables and figures correspond to non-transformed data and, SEM and P-values correspond to the ANOVA analyses of the transformed data. The percentage of each general activity was calculated, and the average by day, pen, and scan obtained. Then, these data were transformed into natural logarithms to achieve a normal distribution. The frequency of each social behavior was calculated by summing by day, pen, and scan, and transformed into the root of the sum of each activity plus 1 to achieve a normal distribution. The ANOVA analysis was performed with transformed data, and the means shown in the tables correspond to the back transformed data.

Performance, eating behavior, animal behavior and concentrate intake were analyzed using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC). The model included initial BW as a covariate, treatment, period (14-d period), and the interaction between treatment and period and fattening cycle (block), as fixed effects, and the interaction between period and pen and the 3-way interaction between pen, period and treatment as random effects. Period was considered a repeated factor, and for each analyzed variable, animal nested within the interaction between treatment and pen (the error term) was subjected to 3 variance-covariance structures: compound symmetry, heterogeneous compound symmetry, autoregressive order one, heterogeneous

autoregressive, and unstructured. The diagonal elements of the UN structure were examined to detect signs of heterogeneous variances across time. Heterogeneity was not detected for any of the variables analyzed. The covariance structure that yielded the smallest Schwarz's Bayesian information criterion was considered the most desirable analysis. The covariate*trt has been checked and the term was removed from the model when not significant. Hot carcass weight was analyzed using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC) including initial BW as covariate, treatment and fattening cycle as fixed effects, and pen as a random effect.

For the analyses of categorical variables (carcass classification, rumen health parameters, hepatic abscesses, and percentage of meal size above 750 g) an independent Chi-square-test was used.

Differences were declared significant at $P < 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

3. Results

3.1. Animal health

Five animals did not finish the study due to health problems; 4 animals from the C group were removed from the study before day 168 because of chronic health problems (lameness and weight loss), and 1 animal from the BF group which had a leg lesion. All the data from these animals were removed from databases. Additionally, the data from 3 animals (1 from the C group and 2 from BF group) which finished the study, were also removed from the databases due to chronic health processes (lameness and bloat).

3.2. Intake and eating pattern

Daily concentrate intake was lesser ($P < 0.05$) for BF group (6.65 ± 0.065 kg of DM/d) compared with C group (6.82 ± 0.065 kg of DM/d) throughout the study (data not shown in the tables; results are presented divided in growing and finishing period). During

Table 3. Performance, concentrate intake, and eating behavior of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation from 4 to 9 mo of age.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	150	148	0.2	<0.01		
Initial BW, kg	195	195	0.7	0.88		
Final BW (112 d of study), kg	387	385	1.9	0.34		
ADG, kg/d	1.72	1.70	0.030	0.59	<0.01	0.96
Concentrate efficiency, kg/kg	0.27	0.28	0.044	0.81	<0.01	0.89
Concentrate DM intake						
Mean, kg/d	6.4	6.3	0.06	0.10	<0.01	0.70
CV, %	17.5	18.0	0.87	0.71	<0.01	0.30
Daily meals						
Mean, number	10.2	9.9	0.29	0.57	<0.01	0.78
CV, %	19.8	19.9	0.43	0.77	<0.01	0.08
Meal size, DM basis						
Mean, kg/meal	668.1	668.8	19.94	0.98	<0.01	0.95
CV, %	22.0	21.7	0.67	0.76	<0.01	0.14
Meal duration						
Mean, min/meal	5.3	5.3	0.29	0.94	<0.01	0.98
CV, %	27.8	26.2	1.24	0.40	0.08	0.30
Total daily meal duration, min						
Mean, min/d	50.2	49.2	1.55	0.66	<0.01	0.66
CV, %	24.5	23.5	1.36	0.61	0.55	0.26
Inter-meal duration						
Mean, min/inter-meal	147.3	151.9	4.06	0.44	<0.01	0.95
CV, %	22.5	22.8	0.73	0.78	<0.01	0.08
Meal eating rate, DM basis						
Mean, g/min	159.4	159.5	7.96	0.99	<0.01	0.58
CV, %	51.1	45.9	4.04	0.38	<0.01	0.35

[1] Control = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

the growing period daily concentrate intake tended to be lesser (P = 0.10) for BF group (6.27 ± 0.060 kg of DM/d) than for C group (6.42 ± 0.060 kg of DM/d) (**Table 3**); however, this difference disappeared in the finishing period (7.51 ± 0.109 kg of DM/d) (**Table 4**).

Table 4. Performance, concentrate intake, and eating behavior of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation from 9 to 11 mo of age.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial BW, kg	387	385	1.9	0.34		
Final BW (112 d of study), kg	476	467	3.0	0.05		
ADG, kg/d	1.55	1.46	0.065	0.35	<0.01	0.65
Concentrate efficiency, kg/kg	0.19	0.18	0.051	0.78	<0.01	0.60
Concentrate DM intake						
Mean, kg/d	7.6	7.4	0.11	0.19	0.30	0.49
CV, %	18.6	17.3	1.41	0.51	0.03	0.47
Daily meals						
Mean, number	10.3	10.6	0.35	0.61	0.16	0.92
CV, %	19.5	19.2	0.66	0.71	<0.01	0.06
Meal size, DM basis						
Mean, kg/meal	782.9	752.8	24.75	0.41	0.08	0.99
CV, %	21.5	20.1	0.97	0.31	0.01	0.18
Meal duration						
Mean, min/meal	4.1	4.2	0.28	0.83	<0.01	0.68
CV, %	29.8	27.8	1.66	0.42	0.06	0.98
Total daily meal duration, min						
Mean, min/d	40.8	42.4	2.12	0.61	<0.01	0.20
CV, %	28.3	26.7	1.83	0.54	0.09	0.93
Inter-meal duration						
Mean, min/inter-meal	149.4	145.6	6.70	0.69	0.53	0.98
CV, %	22.6	21.8	0.68	0.45	0.08	<0.01
Meal eating rate, DM basis						
Mean, g/min	242.3	229.2	20.92	0.66	<0.01	0.37
CV, %	48.2	45.3	4.29	0.64	0.20	0.18

[1] Control = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

No interactions between treatment and time were observed (**Table 3** and **4**) in eating pattern parameters analyzed. During growing phase, no differences were observed in the percentage of meal data above 750 g between treatments. However, in the finishing phase (periods 9 to 12), the proportion of meal size values >750 g was higher ($P < 0.05$) in C (57.3%) compared with BF bulls (49.3%).

Table 5. Carcass quality of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation.

	Treatment[1]			*P-value*[2]
Item	Control	BF	SEM	T
Age before slaughter, d	322	324.6	2.95	0.57
Days in study, d	173	175.3	1.66	0.35
BW before slaughter, kg	490	479	3.98	0.06
Carcass weight, kg	258	254	2.31	0.15
Dressing percentage, %	52.6	53.0	0.18	0.42
Fatness[3], %				0.31
1	1.0	0		
2	13.6	8.8		
3	85.2	91.1		
Conformation[4], %				0.62
R	3.7	6.3		
O	58.0	51.9		
P	34.3	41.8		

[1] Control = non-supplemented, BF = concentrate supplemented with citrus flavonoids.

[2] T = treatment effect.

[3] The carcass fat cover classification, according the EU Regulation No. 1208/81, which utilizes a classification system by numbers, 1.2.3.4.5, where 5 explains a very high degree of covering fat and heavy fat deposits in the thoracic cavity, and 1 is classified as low degree, with no fat cover.

[4](S)EUROP categories described by the EU Regulation No. 1208/81 and 1026/91, the conformation of carcasses is classified as "E" when corresponds to an excellent conformation, "U" to very good conformation, "R" to good conformation, "O" to fair conformation, and "P" to a poor conformation.

3.3. Performance and Carcass Quality

No differences were found for ADG during the growing phase (1.71 ± 0.030 kg/d) nor in finishing period (1.50 ± 0.065 kg/d). However, final BW was higher for C bulls (476.2 ± 3.00 kg) than for BF group (467.8 ± 3.00 kg). Concentrate efficiency for growing (0.27 ± 0.044 kg/kg) and finishing period (0.19 ± 0.051 kg/kg) was not affected by treatment (**Table 3** and **4**). Slaughter BW tended (P = 0.06) to be higher for C group (489.7 ± 3.98 kg) compared with BF group (479.3 ± 3.98 kg), although this difference disappeared for HCW (256.1 ± 2.31 kg) (**Table 5**). Carcass quality data are presented in **Table 5.** Dressing percentage (52.85 % ± 0.182), carcass conformation and fatness were not affected by treatment.

3.4. Animal Behavior

General Activities. General activities are showed in **Table 6**. During the growing phase (from 0 d to 112 d of the study), no differences were found in the percentage of animals per pen standing, lying, drinking, and ruminating throughout the visual observation period (2 h). The proportion of animals eating straw and concentrate was higher ($P < 0.01$ and $P < 0.001$, respectively) for BF bulls (18.72 ± 1.81% and 5.97 ± 0.06%, respectively) compared with C bulls (15.36 ± 1.81% and 5.68 ± 0.06%, respectively during this phase.

Table 6. Percentages of general activities (%) of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
Growing						
Standing	72.2	74.7	2.38	0.25	<0.01	0.48
Lying	27.8	25.3	2.38	0.27	<0.01	0.15
Eating concentrate	5.7	6.0	0.06	<0.01	<0.01	0.26
Eating straw	15.4	18.7	1.81	<0.01	<0.01	0.19
Drinking	1.9	1.6	0.24	0.52	0.73	0.85
Ruminating	12.0	12.7	0.61	0.32	0.05	0.17
Finishing						
Standing	75.7	71.7	4.37	0.40	0.73	0.49
Lying	24.3	28.2	4.57	0.15	0.59	0.49
Eating concentrate	5.3	6.1	0.33	<0.01	0.77	0.66
Eating straw	10.9	15.0	4.05	<0.05	0.13	0.17
Drinking	2.0	1.7	0.72	<0.05	0.06	0.64
Ruminating	8.2	11.4	1.95	0.37	0.19	0.76

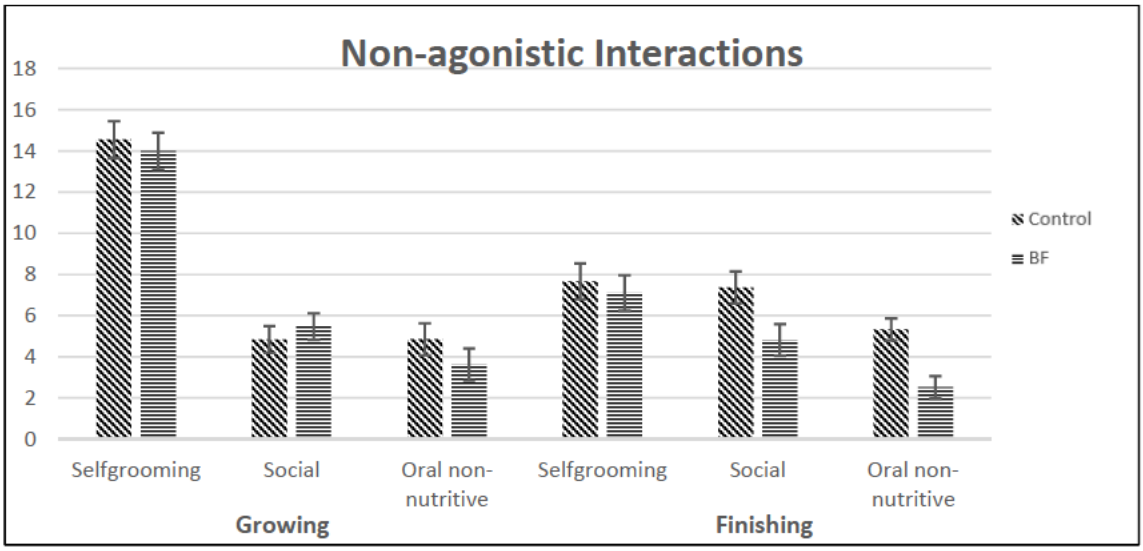
[1] C = control, BF = concentrate supplemented with citrus flavonoids.

[2] T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.

[3] SEM = standard error of the means of the log-transformed data.

During the finishing phase, for the visual observation period (2 h) no differences were observed in the proportion of animals per pen standing, lying, and ruminating. As observed in the growing phase, the proportion of animals per pen eating concentrate was higher ($P < 0.01$) in BF bulls (6.10 ± 0.33%) than in C bulls (5.30 ± 0.33%), and a higher ($P < 0.05$) proportion of animals was eating straw in BF bulls (14.96 ± 4.05%) compared with C bulls (10.89 ± 4.05%). Otherwise, proportion of animals drinking water was lesser ($P < 0.05$) for BF bulls (1.59 ± 0.57%) than for C bulls (1.98 ± 0.57%) in this phase.

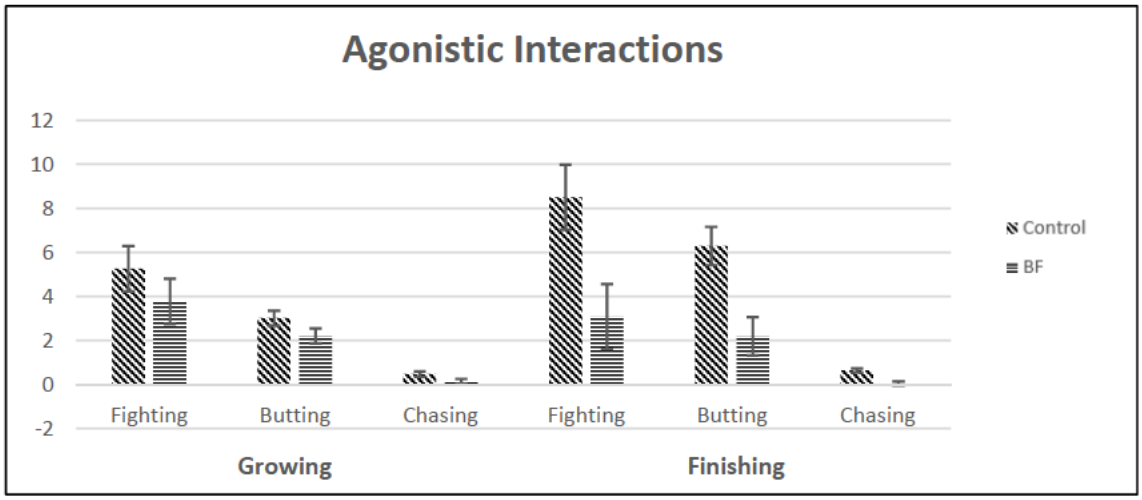
Figure 1. Non-agonistic interactions of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation.



Active Behavior. In the growing phase, during the visual scan observation period of 2 h, no differences were observed for self-grooming and social behavior (14.27 ± 0.89 times/15 min and 5.16 ± 0.64 times/15 min, respectively) between treatments. Bulls of the C group exhibited more ($P < 0.05$) oral non-nutritive behaviors (4.85 ± 0.78 times/15 min) than BF bulls (3.62 ± 0.78 times/15 min) (**Figure 1**). All behaviors related to agonistic interactions were statistically different during this phase (**Figure 2**). The frequency of fighting behaviors was higher ($P < 0.05$) in C bulls (5.25 ± 1.03 times/15 min) than in BF bulls (3.77 ± 1.03 times/15 min). Butting tended to be higher ($P = 0.09$) for C group (3.01 ± 0.35 times/15 min) compared with BF group (2.21 ± 0.35 times/15 min), and an interaction ($P = 0.05$) between treatment and day was observed for this behavior. Displacement interactions were lesser ($P < 0.05$) exhibited by C group (0.18 ± 0.09 times/15 min) compared with BF group (0.27 ± 0.09 times/15 min). Chasing and

chasing-up interactions were higher ($P < 0.01$ and $P < 0.05$, respectively) in the C bulls (0.48 ± 0.12 times/15 min and 0.11 ± 0.05 times/15 min, respectively) than in the BF group (0.14 ± 0.12 times/15 min and 0.02 ± 0.05 times/15 min, respectively), but these behaviors were occasionally exhibited. No differences in sexual behaviors (flehmen, attempt to mount, complete mounts) were observed in this phase (**Figure 3**).

Figure 2. Agonistic interactions of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation.



During the finishing phase (from 113 d to 168 d), no differences were observed for self-grooming behavior (7.39 ± 0.88 times/15 min) between treatments, whilst social and oral behaviors were higher ($P < 0.01$ and $P < 0.001$, respectively) in bulls of the C group (7.37 ± 0.76 times/15 min and 5.33 ± 0.54 times/15 min, respectively) compared with BF bulls (4.81 ± 0.76 times/15 min and 2.52 ± 0.54 times/15 min, respectively) (Figure 1). Regarding agonistic behavior (Figure 2), fighting and butting interactions were higher ($P < 0.001$ and $P < 0.001$, respectively) in C group (8.50 ± 1.47 times/15 min and 6.29 ± 0.87 times/15 min, respectively) than in BF bulls. Although chasing interactions occasionally occurred, bulls from the C group (0.64 ± 0.09 times/15 min) exhibited higher ($P < 0.001$) interactions than BF bulls (0.04 ± 0.09 times/15 min). Flehmen and complete mounts were higher ($P < 0.01$ and $P < 0.05$, respectively) in C bulls (4.35 ± 0.76 times/15 min and 1.81 ± 0.29 times/15 min, respectively) than in BF bulls (2.60 ± 0.76 times/15 min and 0.69 ± 0.29 times/15 min, respectively), whereas attempt to mount interactions tended to be higher ($P = 0.10$) in bulls of the C group (2.02 ± 0.57 times/15 min) compared with BF group (0.96 ± 0.57 times/15 min) (**Figure 3**).

Figure 3. Sexual interactions of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation.

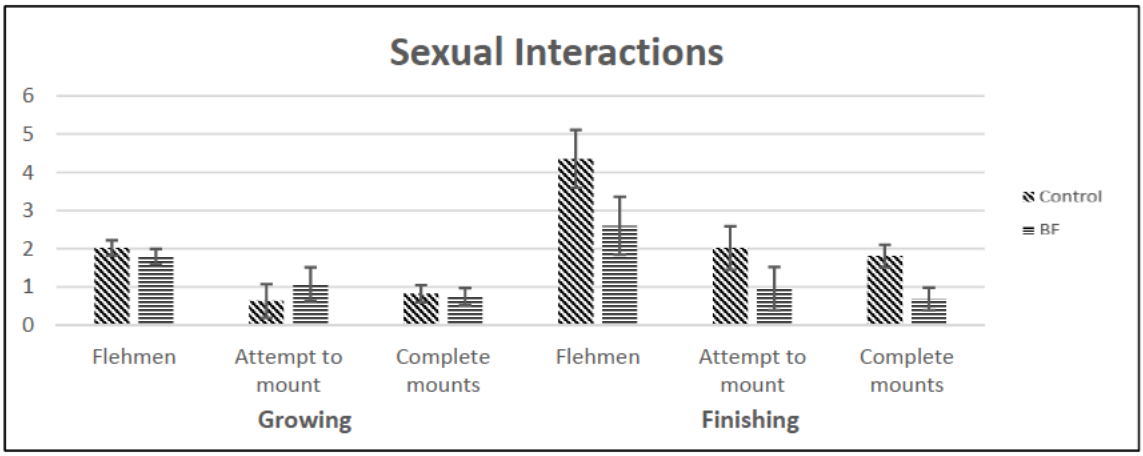


Table 7. Macroscopically observations of the rumen of Holstein bulls fed high-concentrate diets with or without citrus flavonoid supplementation.

	Treatment[1]		*P*-value[2]
Item	Control	BF	
Color of the rumen[3]			0.06
3	42.7	44.3	
4	47.6	54.4	
5	9.8	1.3	
Papillae clumping			0.66
Yes	43.9	40.5	
No	56.2	59.5	
Baldness region			0.01
Yes	67.1	48.1	
No	32.9	51.9	
Liver abscess[4]			0.26
None	78.3	75.6	
A	13.0	22.2	
A-	2.2	-	
A+	2.2	2.2	
Inflammation	4.4		

[1] Control = non-supplemented, BF= concentrate supplemented with citrus flavonoids.

[2] T = treatment effect.

[3] Adapted from Gonzalez et al. (2001): Rumen color: 1= white; 5 = black.

[4] Adapted from Nocek et al. (1984).

3.5. Macroscopic Rumen Evaluation and Liver Abscesses

At the slaughterhouse, color of rumen wall tended (P = 0.06) to be lighter for BF bulls (1.27% classified as color "5") compared with C (9.76% classified as color "5"). Baldness areas presence in the rumen were lesser (P = 0.01) in BF group (48.1%) than in C (67.1%) (**Table 7**). No differences were observed for liver abscesses between treatments at the slaughterhouse (**Table 7**).

4. Discussion

4.1. Intake, eating pattern and performance

Bulls supplemented with flavonoids reduced concentrate intake throughout the study compared with control group, and surprisingly, eating pattern parameters did not differed between treatments. As concentrate intake is the consequence of the meal size and daily number of visits to the feeder, these parameters were more deeply studied. When meal sizes above 750 g were analyzed, no differences were observed in the growing phase (from 0 d to 112 d) between treatments. Contrary, during finishing phase (from 113 d to 168 d), the proportion of meal size values > 750 g was higher (P < 0.05) in C (57.3%) compared with bulls supplemented with flavonoids (49.3%). Therefore, supplementing with BF reduced the percentage of large meal sizes in this phase. The question is how this supplementation with citrus flavonoids could reduce large meal sizes during the finishing phase. There are two hypothetical pathways based on literature.

First, naringin is the main flavonoid of Bioflavex® CA. This glycosylated flavanone is responsible of the typical bitterness in some citrus fruits (Ribeiro et al., 2008). Taste is an important source of information about food composition for animals, and bitter taste has been often related to the presence of toxins (Favreau et al., 2010; Ginane et al., 2011), and this taste is considered as a negative value (Favreau et al., 2010). But herbivores present a high bitter threshold, being more tolerant to this taste than other mammals (Glendinning, 1994). Moreover, in this study meal size exhibited no differences during the growing phase between treatments, with the same content of naringin than in the finishing phase. Thus, bitter taste of citrus flavonoids probably is not the cause of meal size reduction observed in the finishing phase of this study.

Second, previous research has shown an increase in molar proportions of propionate in the rumen of cannulated heifers supplemented with flavonoids (Balcells et al., 2012). According to these results, Seradj et al. (2014) observed that citrus flavonoids increased propionate to detriment of acetate proportion in rumen liquor from steers fed high concentrate diets in an *in vitro* study. Propionate plays a key role as a regulator of feed intake in ruminants fed high-starch diets (Bradford and Allen, 2007). Oba and Allen (2003) found that an intra-ruminal infusion of sodium propionate decreased dry matter intake of lactating cows by decreasing meal size. Propionate produced in the rumen is quickly absorbed during the meal, and acts as an important hypophagic signal in the liver, being the primary signal to stimulate satiety in ruminants fed high-starch diets (Allen et al., 2009 and 2012). Therefore, it could be hypothesized that flavonoids supplementation in bulls could reduce large meal sizes by increasing propionate production into the rumen within the timeframe of the meal.

Regarding the number of visits to the feeder, it was stable throughout the study for bulls of the Control group (10.2 and 10.3 visits/d for growing and finishing phase, respectively). In bulls supplemented with flavonoids, a numerically increase in the number of visits to the feeder during the finishing phase (from 9.9 in the growing phase to 10.6 visits/day in the finishing phase) was observed. Devant and Bach (2017) have reported that steers performing small meal sizes increase the number of visits to the feeder. In this study, in agreement to this observation, bulls supplemented with flavonoids had lesser percentage of meal sizes above 750 g in the finishing phase, and this could explain a numerical increase in the number of visits to the feeder during this phase compared with the growing phase. Nevertheless, this increase in the number of visits to the feeder has not been sufficiently large to increase feed intake, perhaps because to the single space feeder had limited the access to the feed in BF bulls. Our data support the hypothesis that these animals supplemented with flavonoids could be redirecting their intake behavior towards the straw, and straw feeder occupancy data observed in this study were higher for BF bulls. Thus, the third cause why flavonoids supplementation could decrease concentrate intake in this study, could be related to the reduction of meal size. As BF bulls would need to increase the number of visits to the feeder, the feeder design (single space-feeder) in this case could be limiting the access to the concentrate, decreasing total concentrate intake.

Further research is needed to evaluate all 3 hypothesis about the reduction of concentrate intake due to the flavonoids supplementation, and if theses mechanisms could act synergistically.

Although the reduction in concentrate intake of bulls supplemented with flavonoids, ADG, final HCW and efficiency were not affected.

4.2. Carcass Quality

Even though BW before slaughter tended (P = 0.06) to be higher for control group (489.7 ± 3.98 kg) compared with bulls supplemented with flavonoids (479.3 ± 3.98 kg), this difference was no longer present in HCW (256.1 ± 2.31 kg). Lesser concentrate intake of BF bulls could explain inconsistency between final BW and HCW observed in the present study. Moreover, lesser empty digestive tract weight due to lower daily concentrate intake may also explain that the differences observed in the final BW between treatments disappeared for the HCW. Fitzsimons et al. (2014) found moderate negative correlation between carcass conformation score and residual feed intake of beef bulls fed high concentrate diet. This study (Fritzsimons et al., 2014) reported that bulls consuming less DMI had a lighter reticulo-rumen empty. Thus, small meal sizes performed by bulls supplemented with flavonoids, and reduced concentrate intake, probably could cause a reduction of the digestive tract weight of BF bulls, explaining that no differences in carcass weight between treatments are been observed.

As bulls supplemented with flavonoids had a reduced concentrate intake throughout the study, a poor carcass fatness and conformation could be expected, mainly due to a lower energy intake. However, in the present study, flavonoids supplementation did not affect carcass quality, fatness percentage, or carcass classification (**Table 6**).

4.3. Animal Behavior

4.3.1. General activities.

Throughout the study, bulls supplemented with flavonoids showed higher occupancy of the single space-feeder for concentrate as well as for the collective straw feeder. Thus, these animals dedicated more time to eat when the visual observation

procedure was used, although the total meal duration recorded by the computerized feeder did not differ among treatments, and concentrate intake was lower for the two productive phases. The bulls devoted more time to eat during the morning (Verdú et al., 2015), which could explain the incongruity between visual and computerized feeder observations.

Although straw consumption was not registered during the study, BF bulls occupied during more time the straw feeder, then it could be hypothesized that they ate more straw than C bulls. This observation would be in agreement with Balcells et al. (2012), who observed that heifers supplemented with citrus flavonoids consumed more straw than non-supplemented. Although time devoted to ruminating was not different between treatments, this may be because during visual observations higher number of BF bulls were eating concentrate or straw compared to non-supplemented group, and feeding may exert an inhibitory effect on ruminating behavior (Pearce, 1965; Gordon and Mc Allister, 1970; Geoffroy, 1974; Murphy et al., 1983). Or it may be due to the visual scan procedure, which does not describe total daily ruminating activities.

Non-supplemented animals exhibited higher occupancy of the drinker during the finishing phase. Possibly, the higher feed intake exhibited by these bulls during this phase resulted in a higher water consumption, because dry matter intake and water intake are directly related (MacFarlane and Howard, 1972; Silanikove, 1987).

4.3.2. Social Behavior.

Animal abnormal behaviors are indicative of poor welfare. In cattle, aggressive and oral non-nutritive behaviors have been described as indicators of poor welfare (Gonyou et al., 1994; Devant et al., 2016), frustration and discomfort. Microbiota, inflammation and diet (Haagensen et al., 2014; Wiley et al., 2017), may affect behavior in humans and other animals, and gut-brain-microbiota axis has been proposed as a communication network between brain, digestive system and its microbiota. In this study, C bulls exhibited more ($P < 0.05$) oral non-nutritive behaviors than BF animals. This behavior of licking objects with non-nutritional finality has been described as an abnormal oral behavior in cattle, and a gut dysfunction has been suggested as one of the possible causes (Bergeron et al, 2006). Devant et al. (2016) reported that bulls fed high-concentrate diet without access to straw increased oral behaviors, and this was related to

an increase in rumen lesions, low rumination activity and low pH. In agreement with Devant et al. (2016), supplementation with flavonoids in previous studies has showed an increase in straw consumption and rumen pH (Balcells et al., 2012), in the present study in macroscopic rumen wall extraction indicated that wall was less damaged. Moreover, the reduction of large meal sizes (less pH fluctuations) and the increased time devoted to eat straw (reducing time devoted to perform other behaviors and higher insalivation) in BF bulls during the finishing phase could explain a reduction of these oral behaviors.

Bulls supplemented with citrus flavonoids also exhibited less aggressive behaviors (agonistic interactions), as fighting and butting, and less sexual interactions as well. Devant et al. (2016) observed that diet presentation (pellet or meal) and straw provision (with or without) in cattle fed high-concentrate diets modified the expression of different genes (*ffar3, ppyr1, adra2c, occluding* and tnf_{α}), and suggested that the rumen could be involved in the crosstalk between digestive system and brain modifying animal aggressive and sexual behavior. The expression of the gene *ffar3* is stimulated by VFA, mainly for propionic acid, and this gene stimulates the secretion of serotonin (Evans et al., 2013; Devant et al., 2016). Serotonin, as neurotransmitter, may act as an important link within the gut-brain axis, and has been associated with mood modulation (Evans et al., 2013) and a reduction in aggressive behaviors (Haagensen et al., 2014). Additionally, selective serotonin reuptake inhibitors (which increase extracellular serotonin) have been related to libido reduction and sexual problems in humans (Balon, 2006). In previous studies (Balcells et al., 2012; Seradj et al., 2014) it has been observed that citrus flavonoids increase the proportion of propionic acid in rumen. Data of the present study may support the hypothesis that acid propionic can not only be an important molecule modulating eating behavior of BF bulls, it maybe also related to the reduction in aggressive and sexual interactions of BF bulls by serotonin secretion modulation in the rumen.

Furthermore, Qaisrani et al. (2012) observed that feeding pullets with diluted diets (with different sources of non-starch polysaccharides) reduced feather-pecking behavior and increased feeding time. In the present study, BF bulls dedicated more time to perform eating behaviors (straw) and had numerically lesser eating rate, smaller meal sizes and larger straw feeder occupancy than C bulls during the finishing phase. Thus, it could be

hypothesized that these animals had less time to perform these aggressive and sexual behaviors as they were more occupied with feeding events.

4.4. Macroscopic Rumen Evaluation and Liver Abscesses

The lighter and less baldness areas in the rumen walls observed in BF bulls compared with C bulls may be indicative of better rumen health. This observation could be linked to the anti-oxidant and anti-inflammatory properties of flavonoids protecting the mucosa (Cavia-Saiz et al., 2010; Harborne et al., 2000; Heim et al., 2002; Tripoli et al., 2007). Naringin is rapidly deglycosylated by enzymes to naringenin (Busto et al., 2007), and rumen microflora is capable of anaerobic degradation of naringin to naringenin (Cheng et al., 1970; Simpson et al., 1969). Naringenin acts as a potent antioxidant as well, and its anti-inflammatory effects has been deeply described (Manchope et al., 2017). Thereby, flavonoids could be protecting rumen epithelium and improving macroscopic health parameters studied by their antioxidant and anti-inflammatory properties.

Balcells et al. (2012) found that heifers supplemented with an extract of citrus flavonoids, after inducing acidosis in rumen cannulated animals had an increase in lactating-consuming bacteria *Megasphaera elsdenii* and rumen pH was higher compared with non-supplemented animals. Otherwise, large meal sizes have been related to higher pH fluctuations, which can lead to rumen acidosis and liver abscesses (Fulton et al., 1979; Stock et al., 1987, 1990), and higher eating rate may negatively affect rumen health (Sauvant et al., 1999; González et al., 2008). In this study, bulls supplemented with Bioflavex® CA performed smaller meal sizes than non-supplemented group, and eating rate was numerically lesser during the finishing phase. Thus, these eating pattern modifications could have also improved rumen health in BF bulls compared with C group (González et al., 2012), along with pH and microflora modulation.

Finally, as previously mentioned, BF bulls occupied during more time the straw feeder. Straw ingestion in ruminants stimulates rumination and salivation, and the buffer capacity of saliva results in a higher ruminal pH, which can lead to a healthier ruminal epithelium as well.

5. Conclusions

In conclusion, citrus flavonoid supplementation in bulls fed with a single-space feeder modified the eating pattern reducing large meal sizes that may cause a reduction in feed intake. However, animal performance was not affected. Animals supplemented with citrus flavonoids spent more time eating straw. Citrus flavonoids improved rumen wall health parameters analyzed, maybe because of reduction of large meal sizes, as well as their potential antioxidant and anti-inflammatory properties. Otherwise, citrus flavonoid supplementation reduced agonistic behaviors throughout the study, and sexual interactions during the finishing phase.

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CHAPTER IV

Effects of flavonoids extracted from *Citrus aurantium* on performance, eating and animal behavior, rumen health, carcass quality and rumen gene expression in Holstein bulls fed high-concentrate diets in pellet form.

DRAFT

ABSTRACT

One hundred fifty bulls (183.0 ± 7.53 kg BW and 137 ± 1.8 d of age) were randomly allocated to one of 8 pens and assigned to control (C) or (BF) (*Citrus aurantium*, Bioflavex CA, Interquim S.L., Spain, 0.4 kg per ton of concentrate of Bioflavex CA, 20% naringin). Each pen had one drinker, one separate five-space straw feeder, and one separate three-space feeder where pellet concentrate based on corn, barley, DDG and wheat was offered. Concentrate intake was recorded daily, whilst BW and animal behavior fortnightly. Animals were slaughtered after 168 d of study (12 periods of 14 d), HCW and carcass quality were recorded, rumen was examined macroscopically and ruminal epithelium samples were collected for gene expression analyses. Data were analyzed using a mixed-effects model with repeated measures and categorical data with a Chi-Square. Final BW (434.9 ± 7.60 kg), concentrate intake (6.6 ± 0.09 kg/d) and concentrate FCR (kg of concentrate/ kg of BW) (4.48 ± 0.069 kg/kg) of bulls were not affected by treatment. During the finishing phase, oral non-nutritive behaviors tended to be greater ($P < 0.10$) in C bulls than BF bulls, whilst self-grooming and social behaviors were greater ($P < 0.01$) exhibited by BF bulls compared with C bulls. Additionally, agonistic interactions (fighting, butting, and chasing) and flehmen were greater ($P < 0.05$) performed by C than BF bulls in this finishing phase. Throughout the study, BF bulls spent more time ruminating ($P < 0.01$), and during the growing phase also devoted more time to eat concentrate ($P < 0.01$) compared with C bulls. The degree of carcass fat cover was affected by treatment ($P < 0.05$). At the slaughterhouse, color of rumen wall was lighter ($P = 0.05$) for BF compared with C bulls. Furthermore, whereas the gene expression of *bitter taste receptor 16* and pro-inflammatory molecules like cytokine *IL-25*, *β-defensin* tended ($P < 0.10$) to be greater in the rumen epithelium of BF than in C bulls, the relative gene expression of *pancreatic polypeptide receptor 1* and *tumor necrosis factor alpha* were greater ($P < 0.05$ and $P < 0.01$, respectively) for BF bulls compared with C bulls. In conclusion, supplementation with flavonoids extracted from *Citrus aurantium* in bulls fed high-concentrate in pellet form reduces agonistic interactions and oral non-nutritive behaviors, increasing time devoted to eat concentrate during the growing phase and ruminating activity throughout the study. Moreover, flavonoid supplementation modifies the expression of genes in the rumen epithelium that could be related with inflammation and animal behavior modulation.

Keywords: bulls, flavonoids, performance, behavior, rumen inflammation, bitter taste receptors.

1. Introduction

Last years, the study of different plant secondary metabolites in beef cattle to improve animal health, productivity and efficiency has showed promising results as natural alternatives to chemical, drugs and growth promoters (Durmic and Blache, 2012). These secondary plant metabolites include essential oils, tannins, saponins, and flavonoids, among others (Patra and Saxena, 2010; Cobellis et al., 2016a and 2016b; Rira et al., 2019). The exact mode of action of these compounds remains unknown. Citrus flavonoids may affect rumen microbiota and fermentation (Balcells et al., 2012, Seradj et al., 2014) or might be directly interacting with several receptors in the gut modifying eating and animal behavior in bulls fed high-concentrate diets. In a previous study (Chapter III) was observed that supplementing a pure extract from bitter orange (*Citrus aurantium*) rich in naringin (Bioflavex CA, Interquim, S.A., Spain) throughout the fattening period of Holstein bulls fed high-concentrate diets, reduced the large meal sizes (> 750 g/ meal) performed by the animals during the finishing phase. Furthermore, bulls supplemented with these citrus flavonoids modified their eating pattern and spent more time occupying concentrate and straw feeders, although final BW and HCW were numerically reduced. This previous study (Chapter III) was performed by a single space feeder in order to record the individual eating pattern of the animals. As eating behavior can be modified by diet and housing conditions (feeder, pen density) it was hypothesized that the single-space feeder could have been limiting the access to the concentrate of the bulls supplemented with flavonoids, impeding these animals to increase the number of visits to the feeder to compensate the reduction in meal size observed. Thus, the present study was carried out in a commercial farm with multiple-space feeders to analyze the possible interaction between feeder-space availability and citrus flavonoids supplementation.

Therefore, the possible mode of action whereby the citrus flavonoids could modify eating and animal behaviors different possible mechanisms should be explored. On the one hand, citrus flavonoids modify ruminal fermentation affecting VFA production in the rumen, and increasing molar proportions of propionic acid (Balcells et al., 2012; Seradj et al., 2014). Propionic acid plays a key role in the regulation of feed intake in ruminants fed high-concentrate diets, being an hypophagic cue (Oba and Allen, 2003; Bradford and Allen, 2007). On the other hand, naringin is conferring the typical bitter taste to citrus

fruits (Ribeiro et al., 2008), and this bitter taste could be identified as a negative value by animals (Favreau et al., 2010; Jaggupilli et al., 2016). A great number of studies carried out last decades have found that different taste receptors (sweet, bitter, soar, salty and umami) are expressed in gastrointestinal tract of humans and different species (Behrens and Meyerhof, 2011; Breer et al., 2012). Consequently, bitter taste receptors (*TAS2R*) could be involved in eating pattern modulation observed when concentrate was supplemented with citrus flavonoids. Thus, both ruminal propionic acid and bitter taste of citrus flavonoids could be involved in the eating pattern modulation observed when bulls were supplemented with citrus flavonoids.

In the previous study (Chapter III) flavonoids supplementation improved rumen wall health parameters analyzed, and reduced agonistic behaviors and sexual interactions in bulls. Recently, gut-brain-microbiota axis has been proposed as a communication network between brain, digestive system and its microbiota, being involved in the gastrointestinal homeostasis maintenance (Haagensen et al., 2014; Carabotti et al., 2015; Wiley et al., 2017). Furthermore, some studies have suggested that inflammation and diet may be implicated in animal behavior modulation (Haagensen et al., 2014) through the gut-brain axis network. In beef cattle, Devant et al. (2016) pointed out that some of these gut-brain crosstalk mechanisms could occur in the rumen, after observing the effects of different diets over the gene expression of some receptors involved in this network.

Thus, the present study was designed to explore the effects of citrus flavonoids supplementation in bulls fed high-concentrate diets on performance (concentrate consumption, growth and concentrate efficiency), carcass characteristics, rumen wall health and animal behavior in commercial conditions (multispace feeders). Additionally, the expression of genes involved in the gut-brain axis crosstalk, such as bitter taste receptors, some neurotransmitters receptors and different inflammation regulators will be deeply investigated in rumen epithelium to highlight the mechanisms involved in eating and animal behavior modulation when bulls are supplemented with citrus flavonoids.

2. Materials and methods

2.1. Animals, feeding, housing, and experimental design

This study was conducted in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado, 2013). One hundred fifty Holstein bulls (183.0 ± 7.53 kg of body weight (BW) and 137 ± 1.8 d of age) were fattened under commercial conditions in a farm (Granja l'Alsina, L'Alsina, Lleida). The whole study lasted 168 d, and was divided into growing (0 to 112 d) and finishing (113 to 168 d) phase. Animals were randomly allocated in one of eight 8 pens, and assigned to one of the two treatments (4 pens per treatment and 18 animals/ pen), either control (C) or supplemented (BF) with 0.04 % of bitter orange extract (*Citrus aurantium*) of the whole fruit rich in naringin (>20%) (Bioflavex CA, Interquim, S.A., Barcelona, Spain). Bioflavex was incorporated into the concentrate during the concentrate manufacturing. The pelleting process started with a grinding process of the ingredients through a roller mill with 2.75 mm sized screen openings. After that, as described by Verdú et al. (2017), a steam-conditioning was used, applying 80ºC of temperature and 0.5 min of retention time, before pelleting. The pellet mill was equipped with a die ring (3.5 mm diameter holes and 70 mm thickness), and after pelleting the exit temperatures ranged ± 10ºC in relation to conditioning temperature. The pellet die knife was set at 10 mm from the die face and pellets were pneumatically transferred to a cyclone cooler with a retention time of 20 min. Finally, pellets were screened on a 2-screen rotex. The manufactured pellets had a uniform diameter (3.5 mm) and length (10 mm). Concentrates were manufactured from a 9,000 kg master-batch, of which 4,500 kg were C, and the other 4,500 kg BF. Each treatment concentrate was transported to the farm with the same truck, and stored into two different silos under the same conditions.

Pens were totally covered (12 m x 6 m) and were deep-bedded with straw and equipped with a three-space feeder (1.50 m length, 0.40 m width, 1.50 m height, and 0.35 m depth). The feeder of each pen weighed the concentrate continuously as described by Verdú et al. (2017), and these data were recorded to calculate concentrate consumption by pen. Pens were also equipped with one drinker (0.30 m length, 0.30 m width, 0.18 m depth). Straw was offered *ad libitum* in a separated straw five-space feeder (3.60 m length, 1.10 m wide, and 0.32 m depth), and every time it was replaced it was recorded to estimate

the total straw consumption. As straw was also used for bedding, these data are only an estimation.

Table 1. Ingredients and nutrient composition of the feed concentrates.

Item	Growing[1]	Finishing[2]
Ingredient, g/ kg		
Corn grain meal	399.7	450.9
Barley grain meal	179.8	155.5
DDGs	179.8	150.2
Wheat	109.7	110.3
Beet pulp	73.9	80.2
Palm oil	20.0	25.0
Calcium carbonate	15.5	12.8
Urea	8.0	4.0
Sodium bicarbonate	5.0	4.0
Dicalcium phosphate	3.6	3.1
Vitamin premix	3.0	2.0
Salt	2.0	2.0
Nutrient		
ME, Mcal/kg DM	3.30	2.97
CP, g/ kg DM	157	140
Ether extract, g/ kg DM	58	61
Ash, g/ kg DM	56	50
NFD, g/ kg DM	178	172
NFC, g/ kg DM	551	577

[1] from 0 to 112 days of the study.
[2] from 113 days to the end of the study.

2.2. Feed consumption and performance

Animals were fed a commercial concentrate in meal form, formulated to cover their nutritional requirements (FEDNA, 2008). The first 112 d of the study, animals were fed a grower concentrate formula, and between 112 d to the end of the study, animals were fed a finisher concentrate. Ingredients and nutritional composition of the concentrates are showed in **Table 1**. Throughout the study, animals had *ad libitum* access to wheat straw (3.5 % CP, 1.6 % ether extract, 70.9 % NDF, and 6.1 % ash; DM basis) and fresh water.

Animals were weighed individually every 14 d throughout the study in 12 experimental periods of 14 d. As already mentioned, during the 8 first periods (from 1 d to 112 d) the animals consumed the growing concentrate and during the last 4 periods (from 113 d to 168 d) and during the days before slaughter animals consumed the finishing concentrate (see Table 1). After 168 d of study bulls were transported to the slaughterhouse (Escorxador del Grup Alimentari Guissona, Guissona, Spain), located 15 km from the farm. Animals were slaughtered in two weeks, 4 pens per week, two pens from C and two from BF bulls each week. The time waiting before slaughter was less than 6 h. Animals were weighed before loading. They were slaughtered by commercial practices and following the EU Regulation 1099/2009 on the protection of animals at the time of killing or slaughtering.

2.3. Animal behavior

A visual scan procedure at days 13, 28, 44, 56, 72, 83, 100, 114, 128, 143, 153, and 171 of the study was performed to study the general activity (standing, lying, eating, drinking, and ruminating) and social behavior (nonagonistic, agonistic, and sexual interactions) of the animals in every pen. Social behavior activities recorded are described in **Table 2, Chapter III**.

General activities recorded were: consumption (when an animal had its head into the feeder and was engaged in chewing) of concentrate, and straw, drinking (when an animal had its mouth in the water bowl), ruminating (including regurgitation, mastication and swallowing of the bolus). Also, postures such as standing or lying (sternal recumbence with all legs folded under the body with the head down or up) were recorded. The visual observation was made for 2 pens at the same time from 8:00 to 10:30 h am, as described by Verdú et al. (2015). General activities were scored using 3 scan samplings of 10 s at 5 min intervals, and social behavior was scored during three continuous sampling periods of 5 min. This scanning procedure of 15 min was repeated twice consecutively in each pen, starting randomly in a different pen every scanning day. This method describes a behavior exhibited by an animal at a fixed time interval (Colgan, 1978).

2.4. Carcass quality

After slaughtering, HCW was registered for every animal. Dressing percentage was calculated by dividing HCW by BW recorded before slaughtering. And, following the (S)EUROP categories described by the EU Regulation No. 1208/81 and 1026/91, conformation of carcasses was classified, where "E" corresponded to an excellent conformation, "U" to very good conformation, "R" to good conformation, "O" to fair conformation, and "P" to a poor conformation. The fat cover was classified according the EU Regulation No. 1208/81, which utilizes a classification system by numbers, 1.2.3.4.5, where 5 explains a very high degree of covering fat and heavy fat deposits in the thoracic cavity, and 1 is classified as low degree, with no fat cover.

2.5. Rumen and liver macroscopic evaluation and sample collection

Rumen and liver of every animal were macroscopically evaluated at the slaughterhouse. Rumens were classified depending on the color by a visual evaluation, from 1 to 5, being "5" a black colored rumen and "1" a white colored rumen (González et al., 2001). They were also divided into areas according to Lesmeister et al. (2004) to examine the presence of ulcers, baldness regions, and of clumped papillae (Nocek et al., 1984). Liver abscesses were classified according to Brown et al. (1975).

Additionally, a liquid sample from rumen was obtained from homogeneous contents strained with a cheesecloth from 18 animals randomly selected from two pens per treatment, immediately following slaughter. Following the procedures of Jouany (1982), 4 mL of ruminal fluid was mixed with 1 mL of a solution containing 0.2% (wt/wt) mercuric chloride, 2% (wt/wt) orthophosphoric acid, and 2 mg/mL of 4-methylvaleric acid (internal standard) in distilled water, and stored at -20ºC until subsequent VFA analysis. Also, a 1-cm2 section of rumen wall (left side of the cranial ventral sac) was sampled and papillae were excised before rinsed 2 times with chilled PBS after sampling and immediately incubated in RNA-later (Invitrogen, Madrid, Spain) to preserve the RNA integrity. After 24 hours of incubation with RNA later at 4 ºC, the liquid was removed and tissue was frozen at -80 ºC until further RNA extraction and subsequent gene expression analysis.

2.6. Biological and chemical analyses

During the study, samples of concentrate were collected at d 0, 42, 84, 126, and 168 d. and analyzed for DM (method 925.04; AOAC, 2005), ash (method 642.05; AOAC, 2005), CP by the Kjeldahl method (method 988.05; AOAC, 2005), ADF and NDF according to Van Soest et al. (1991) using sodium sulfite and alpha-amylase, and EE by Soxhlet with a previous acid hydrolysis (method 920.39; AOAC, 2005).

Naringin was determined for every sample of concentrate (C and BF) as a Bioflavex CA marker for BF group, and was used as a marker confirming adequate inclusion of citrus flavonoid extract in the diets by Laboratory of Interquim S.A. Internal method for naringin quantification using HLPC developed by Interquim S.A. was used (Paniagua et al., 2018).

Ruminal VFA concentration was determined with a semicapillary column (15 m × 0.53 mm ID, 0.5 µm film thickness, TRB-FFAP, Teknokroma, Barcelona, Spain) composed of 100% polyethylene glycol (PEG) esterified with nitroterephtalic acid, bonded and crosslinked phase (method number 5560*;* APHA–AWWA–WPCF, 2005), using a CP-3800-GC (Varian, Inc., Walnut Creek, CA).

Table 2. Sequence, annealing temperature (At), concentration (µM), efficiency (%) and amplicon size (bp) of the primers used for qPCR.

	Fw Primer	Rv Primer	At	µM	bp	Efficiency
RPS9	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC	57	0.125	63	2.04
TAS2R7	TGGGGTGTTTGGTCTTCTCG	GGCAATGAAAAGGAGGAGGAATG	60	0.25	218	1.96
TAS2R16	GCTTGAGAGACTTGAGGCGT	GCCATGAGCAAGACTGTGGA	60	0.25	108	1.96
TAS2R38	AATTTCCGGGACCTGGTGAG	AGCTCAGCGGGTCTTTCATC	60	0.25	151	1.97
TAS2R39	GTGGCGGATTTCTCCTACCC	CACTCTGGCCCAAGGAAACA	60	0.5	105	2.09
LTF	TGAAAGGGGAAGCAGATG	AAGTCCTCACGATTCAAGTT	50	0.5	552	1.98
PPYR1	TGAGGCCATCCCCATTTGTC	CTCAGACTCCTCCACAGGGA	57	0.25	174	2.22
TLR4	TCAGAAACCTCCGCTACCTTG	TTCTGAAAAGAGTTGCCTGCC	55	0.5	117	1.91
FFAR2	CGCTCCTTAATTTCCTGCTG	CAAAGGACCTGCGTACGACT	52	0.5	173	2.03
FFAR3	AAAGCAGCAGTGGCCATGA	GAGGTTTAGCAAGAGCACGTCC	57	0.25	182	1.98
ADRA2C	TGCGCGCCCCGCAGAACCTCTTCCT	ATGCAGGAGGACAGGATGTACCA	59	0.5	403	1.97
CLDN4	CATGATCGTGGCCGGCGTG	AGGGCTTGTCGTTGCGGG	62	0.125	226	1.82
TNFα	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	60	0.5	296	1.89
B-DEF	GGTCACAAGTGGCAGAGGAT	TGGTTGAAGAACTTCAGGGC	60	0.25	152	2.01
CCKBR	TGTGTTGGTTGCCCGTGTAT	AGGCGTAGCTTAGCAAGTGG	60	0.25	114	1.97
IL-25	TAAGGCTGTCACCTTGCCTC	CGAGCCCAACTTCTATCCCC	60	0.25	194	1.89
UXT	TGTGGCCCTTGGATATGGTT	GGTTGTCGCTGAGCTCTGTG	57	0.125	100	2.05
ACTB	CTGGACTTCGAGCAGGAGAT	CCCGTCAGGAAGCTCGTAG	57	0.125	75	1.82
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	52	0.125	67	2.03

For gene expression analyses, total RNA was extracted from rumen wall homogenizing tissues in Trizol (Invitrogen) by Polytron Instrument (IKA, Germany). Isolated mRNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (Takara, Frankfurt, Germany) following the manufacturer's instructions. The RNA purity was assessed by a NanoDrop instrument (ThermoFisher, Madrid, Spain) at 260, 280, and 230 nm. The quantification of the expression of genes at the mRNA level coding for 1) the tight-junction protein Claudin4 *(CLDN4)*; 2) the production, expression, and turnover of neurotransmitters: free fatty acid receptor 2 (*ffr2*) and free fatty acid receptor 3 (*ffr3*), pancreatic polypeptide receptor 1 (*ppyr1)*; actual name neuropeptide Y receptor Y4 [*npy4r*]), and α2-adrenergic receptor subtype C (*adra2c*), cholecystokinin receptor 4 (*cckbr*); 3) pro-inflammatory cytokines TNF-α (*TNFα*) and cytokine IL-25 *(IL-25)*, pattern recognition receptor Toll-like receptor 4 *(TLR4)* and antimicrobial peptides released by intestinal cells (*β-defensins*, and *lactoferrin*); 4) bitter taste receptors type 2 member 7, 16 ,38 and 39 (*TAS2R7, TAS2R16, TAS2R38* and *TAS2R39)* were performed by quantitative PCR (qPCR). The qPCR was performed using gene codifying for Ribosomal Protein Subunit 9 (*RPS9*) as a housekeeping gene, which was checked for stability following Vandesompele et al. (2002) in comparison with genes codifying for β-actin (*ACTB*), ubiquitously expressed Transcript protein (*UXT*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The qPCR conditions for each set of primers were individually optimized (**Table 2**). The specificity of the amplification was evaluated by single band identification at the expected molecular weight in 0.8% DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by amplifying serial 1:10 dilutions of each gene amplicon. A standard curve of crossing point (Cp) versus the logarithm of the concentration was plotted to obtain the efficiency, which was calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8 to 2.2. A total reaction volume of 20 μL was used, containing 50 ng of cDNA, 10 μL of SYBR Premix EXTaq (TliRNAseH) (Takara, Frankfurt, Germany) and the optimized primer concentration for each gene (**Table 2**). The qPCR reactions were performed as follows: an initial denaturing step of 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C, and a final extension of 10 min at 72°C. The resulting Cp values were used to calculate the relative expression of selected genes by relative quantification using a reference gene (housekeeping gene) and a calibrator of control group (Pfaffl, 2004, Eq. [3.5]).

2.7. Calculations and statistical analyses

Only pen was considered the experimental unit and animals within pen were considered sampling units in some parameters.

Concentrate efficiency data were transformed into log to achieve a normal distribution. The means presented in the tables and figures correspond to non-transformed data and, SEM and P-values correspond to the ANOVA analyses of the transformed data. The percentage of each general activity was calculated, and the average by day, pen, and scan obtained. Then, these data were transformed into natural logarithms to achieve a normal distribution. The frequency of each social behavior was calculated by summing by day, pen, and scan, and transformed into the root of the sum of each activity plus 1 to achieve a normal distribution. The ANOVA analysis was performed with transformed data, and the means shown in the tables correspond to the back transformed data.

Unification of performance, animal behavior and concentrate consumption data averaged by pen and period were analyzed using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC). The model included initial BW as a covariate, treatment, period (14-d period), and the interaction between treatment and period, as fixed effects, and the interaction between treatment and pen and the 3-way interaction between treatment, pen, and period as random effects. Period was considered a repeated factor, and for each analyzed variable, animal nested within the interaction between treatment and pen (the error term) was subjected to 3 variance-covariance structures: compound symmetry, autoregressive order one, and unstructured. The covariance structure that yielded the smallest Schwarz's Bayesian information criterion was considered the most desirable analysis.

In the case of rumen gene expression data were transformed into log to achieve a normal distribution. The means presented in the figure correspond to non-transformed data and, SEM and P-values correspond to the ANOVA analyses of the transformed data. Pen were considered the experimental unit and animals as sampling units, and data were analyzed using ANOVA where the model included treatment (as there were no repeated measures) as the main effect. For VFA data, also pen were considered the experimental unit and animals as sampling units, and data were analyzed using ANOVA where the model included treatment (as there were no repeated measures) as the main effect. For

categorical variables analyses (carcass classification, rumen health parameters, hepatic abscesses) a Chi-square-test was used.

Differences were declared significant at $P < 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

3. Results

3.1. Animal health

Three animals from the BF treatment were removed before the end of the study, two of them due to lameness problems, and one due to an accident. One animal from the C treatment was also removed due to lameness problems.

3.2. Intake, performance and carcass quality

Although no statistical differences were found in concentrate intake between treatments throughout the study (**Table 5**), neither during the growing (**Table 3**) nor for the finishing phase (**Table 4**), however a significant interaction between treatment and time was found during the whole study (**Table 5, Figure 1**), and also during growing

Table 3. Performance and concentrate intake for growing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	137.26	137.29	1.810	0.99		
Final age, d	259.26	249.29	1.738	0.99		
Initial BW, kg	182.78	183.23	7.528	0.97		
Final BW (112 d of study), kg	344.55	346.31	7.716	0.87		
CV, %	8.08	7.91	0.446	0.79		
ADG, kg/d						
Mean, kg/d	1.45	1.45	0.018	0.85	<.0001	0.52
CV, %	24.63	26.82	2.181	0.48	<0.10	0.21
Concentrate DM intake						
Mean, kg/d	5.91	5.87	0.090	0.74	<.0001	<0.05
CV, %	13.86	13.24	0.764	0.57	<.0001	0.87
FCR, kg/kg	4.11	4.13	0.047	0.69	<0.01	0.57

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

(**Table 3**) and finishing (**Table 4**) phases. The **Figure 1** represents the mean of the concentrate intake by period throughout this study. During the growing phase (from period 1 to 8), BF bulls had lower concentrate intake than C bulls only in period 6 and in the last period of the finishing phase (period 12). An interaction between period and treatment was found for CV of concentrate intake during the finishing phase (**Table 4**). Thus, the CV was greater for BF than for C bulls during the period 9 but, on the contrary, lesser for BF bulls compared with C bulls in period 11, whereas for period 10 and 12 no differences were found (data not shown). On the other hand, the estimation for straw consumption for the growing phase (0.81 ± 0.065 kg/d and 0.72 ± 0.065 kg/d for C and BF, respectively), and also for finishing phase (1.05 ± 0.129 kg/d and 1.08 ± 0.129 kg/d for C and BF, respectively) was not statistically different (P = 0.91 and P = 0.36, respectively) between treatments neither.

Table 4. Performance and concentrate intake for finishing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	259.26	249.29	1.738	0.99		
Final age, d	306.26	306.28	1.735	0.99		
Initial BW, kg	344.55	346.31	7.716	0.87		
Final BW (168 d of study), kg	433.99	435.88	7.595	0.87		
CV, %	7.94	7.12	0.592	0.36		
ADG, kg/d						
Mean, kg/d	1.56	1.55	0.026	0.83	0.11	0.36
CV, %	33.60	32.62	3.229	0.83	0.11	0.16
Concentrate DM intake						
Mean, kg/d	8.04	7.91	0.108	0.39	<.0001	<0.01
CV, %	13.05	12.40	0.989	0.65	<0.10	0.05
FCR, kg/kg	5.23	5.14	0.122	0.60	0.19	0.68

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

Performance parameters analyzed throughout the study, as ADG, final BW and FCR, did not evince any statistical differences between treatments (**Table 5**), neither during the growing (**Table 3**) nor during the finishing phase (**Table 4**).

Table 5. Performance and concentrate intake for the whole study in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF® CA	SEM	T	Time	T x Time
Initial age, d	137.26	137.29	1.810	0.99		
Final age, d	306.26	306.28	1.735	0.99		
Initial BW, kg	182.78	183.23	7.528	0.97		
Final BW (168 d of study), kg	433.99	435.88	7.595	0.87		
CV, %	7.94	7.12	0.592	0.36		
ADG, kg/d						
Mean, kg/d	1.48	1.48	0.018	0.98	<.0001	0.54
CV, %	27.62	28.76	1.965	0.68	<0.01	0.17
Concentrate DM intake						
Mean, kg/d	6.62	6.54	0.094	0.58	<.0001	<0.01
CV, %	13.59	12.96	0.591	0.46	<.0001	0.64
FCR, kg/kg	4.48	4.47	0.069	0.90	<.0001	0.70

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

Table 6. Carcass quality from Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P-value*[2]
Item	C	BF	SEM	T
Age before slaughter, d	323.19	323.11	1.019	0.96
Days in study, d	186.06	186.04	0.367	0.98
BW before slaughter, kg	461.50	464.71	3.423	0.51
Hot carcass weight, kg	243.11	242.04	2.126	0.72
Dressing percentage, %	52.71	52.11	0.294	0.15
Fatness, %				0.03
1	2.67	1.43		
2	9.33	18.57		
3	88.00	80.00		
Conformation, %				0.22
P	60.00	51.43		
O	40.00	45.71		
R		1.43		
U		1.43		

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect.

Regarding carcass quality, data are presented in **Table 6**. Even though BW before slaughter, hot carcass weight (HCW) and dressing percentage were not affected by treatment, statistical differences were found for carcass fatness classification. Thus, C group had greater ($P < 0.05$) percentage of animals classified with score "3" of fatness degree than BF bulls.

3.3. Animal behavior

Animal behavior data, including general activities along with active behavior, are showed in **Table 7** and **Table 8** for growing and finishing phase, respectively.

General activities. During the growing and finishing phase, in most of the activities registered no statistical differences were observed. The proportion of animals ruminating in BF group was greater ($P < 0.01$) compared with C bulls in the growing and finishing phase, whereas the percentage of animals eating concentrate was greater ($P < 0.01$) for BF compared C bulls for the growing phase.

Active behavior. In the growing phase, self-grooming and social behaviors were greater ($P < 0.01$) exhibited by BF compared with C bulls, whereas C bulls exhibited more ($P < 0.05$) agonistic behaviors as butting, displacement and chasing, than BF bulls. BF bulls also tended ($P < 0.10$) to perform less fighting, whikst flehmen behaviors were greater ($P = 0.05$) in C bulls compared with BF bulls. Sexual behaviors, as attempted and complete mounts were not affected by treatment during this phase.

Regarding the finishing phase, again self-grooming and social behaviors were greater ($P < 0.05$) exhibited by BF than C bulls. During this phase, C group tended ($P < 0.10$) to perform more oral non-nutritive behaviors compared with BF bulls. Agonistic behaviors, as fighting, butting, chasing and displacement, were clearly greater ($P < 0.05$) exhibited in this phase by C group than BF animals. Additionally, C bulls also exhibited greater ($P < 0.01$) flehmen behaviors (**Figure 2**) and tended ($P < 0.10$) to perform more attempted mounts than BF bulls during this finishing phase (**Figure 3**).

Table 7. General activities (%) and social behavior (times/ 15 min) for growing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
General Activity, %						
Standing	78.46	77.79	0.058	0.78	<.0001	0.93
Lying	19.66	20.33	0.191	0.68	<.0001	0.48
Eating concentrate	7.64	9.99	0.060	<0.01	0.05	0.33
Eating straw	11.51	12.81	0.025	0.19	<.0001	0.32
Drinking	1.55	2.63	0.003	0.87	0.84	0.92
Ruminating	7.66	11.37	0.082	<0.01	0.05	0.61
Social behavior, /15 min						
Selfgrooming	19.32	28.95	0.102	<.0001	0.58	0.52
Social	1.96	4.02	0.126	<0.01	0.42	0.62
Oral non-nutritive	0.77	0.59	0.049	0.44	<0.01	0.27
Fighting	9.16	4.91	0.346	<0.10	<.0001	0.76
Butting	2.30	1.18	0.098	0.01	<.0001	0.93
Displacement	0.464	0.214	0.033	<.0001	0.80	0.67
Chasing	0.70	0.20	0.071	<0.05	<0.05	<0.10
Chasing up	0.16	0.05	0.029	0.20	0.45	0.12
Flehmen	2.21	1.55	0.091	0.05	<0.01	0.74
Attempt to mount	2.21	1.44	0.153	0.22	<0.01	0.75
Complete mounts	2.55	2.00	0.091	0.34	<0.05	0.74

1. C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.

2. T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.
3. SEM = standard error of the means of the log-transformed data (general activity) or root transformed data (social behavior).

Table 8. General activities (%) and social behavior (times/ 15 min) for finishing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
General Activity, %						
Standing	67.14	66.41	0.080	0.86	<0.01	0.73
Lying	32.86	32.93	0.168	0.93	<0.01	0.85
Eating concentrate	6.27	8.10	0.060	0.18	0.17	0.48
Eating straw	7.86	8.67	0.065	0.64	<.0001	0.20
Drinking	2.14	2.54	0.004	0.95	0.95	0.94
Ruminating	8.12	14.03	0.072	<0.01	0.14	0.59
Social behavior, /15						
Selfgrooming	10.20	16.90	0.150	<.0001	<.0001	0.62
Social	3.85	5.93	0.210	<0.05	0.54	0.52
Oral non-nutritive	1.38	0.78	0.067	<0.10	<0.01	0.15
Fighting	8.85	3.85	0.256	<0.01	<0.01	0.80
Butting	4.18	1.60	0.214	<0.05	0.69	0.34
Displacement	1.00	0.15	0.070	<.0001	0.23	0.62
Chasing	0.56	0.08	0.050	<0.05	0.32	0.20
Chasing up	0.23	0.05	0.032	0.11	0.39	0.24
Flehmen	5.63	2.35	0.079	<.0001	0.01	0.56
Attempt to mount	1.45	0.10	0.228	<0.10	<0.10	0.49
Complete mounts	2.80	1.58	0.165	0.18	<0.10	0.44

1. C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.

2. T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.

3. SEM = standard error of the means of the log-transformed data (general activity) or root transformed data (social behavior).

3.4. Macroscopic rumen evaluation and liver abscesses

At the slaughterhouse (**Table 9**), rumen wall color of the BF bulls was lighter ($P < 0.05$) compared with C bulls (72.86% vs. 50.67% classified as color ≤ "3" for BF and C bulls, respectively). No differences between treatments in liver abscesses, baldness regions and clumped papillae were observed at the slaughterhouse.

3.5. Rumen pH and VFA concentration at slaughterhouse

Ruminal fermentation parameters data are presented in **Table 10**. Total VFA concentration and pH in the rumen were not affected by treatment. The molar proportion of isovalerate was greater ($P < 0.05$) in BF bulls compared with C bulls, whereas molar proportion of the remaining of VFA analyzed (acetate, propionate, butyrate, valerate, and isobutyrate) were not affected by the treatment. Accordingly, acetate:propionate ratio was also not affected by treatment.

Table 9. Macroscopical observations of the rumen and liver at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]		*P*-value[2]
Item	C	BF	
Color of the rumen[3]			<0.05
2	2.67	2.86	
3	48.00	70.00	
4	48.00	24.29	
5	1.33	2.86	
Papillae clumping			0.60
Yes	40.00	44.29	
No	60.00	55.71	
Baldness region			0.25
Yes	40.00	49.33	
No	60.00	50.67	
Liver abscess[4]			0.41
None	90.67	94.29	
A	2.67	1.43	
A-	4.00	2.86	
A+	1.33	-	
Inflammation	1.33	1.43	

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect.
[3]Adapted from Gonzalez et al. (2001): Rumen color: 1= white; 5 = black.
[4]Adapted from Nocek et al. (1984).

3.6. Expression of genes in the rumen epithelium

The results of the relative gene expression at mRNA level in the rumen epithelium are showed in **Figure 4**. The supplementation with citrus flavonoids only affected statically the expression of the *TAS2R16*, that tended (P < 0.10) to be greater expressed in BF bulls than in C, whereas the rest of *TAS2R* analyzed (*TAS2R7*, *TAS2R16*, *TAS2R38*, and *TAS2R38*) were not affected by treatment. Regarding the relative expression of receptors related with the neurotransmitter signaling, only *ppyr1* differed among treatments, being greater (P < 0.05) expressed for BF bulls than for C bulls. Additionally, the relative expression of the receptors related with inflammation like cytokine *IL-25* and *β-defensin* tended (P < 0.10) to be also were greater expressed in BF compared with C bulls, whereas *TNFa* was greater (P < 0.01) expressed in BF bulls than in C bulls.

Table 10. Ruminal fermentation parameters at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P-value*[2]
	C	BF	SEM	
pH	5.91	5.90	0.142	0.95
Total VFA, m*M*	111.1	121.7	8.98	0.41
Individual VFA, mol/100 mol				
Acetate	53.1	53.1	1.50	0.98
Propionate	35.9	36.2	1.63	0.90
Isobutyrate	0.7	0.6	0.11	0.40
n-butyrate	7.0	7.1	0.32	0.77
IsoValerate	1.5	0.9	0.16	0.03
Valerate	1.9	2.0	0.13	0.35
Acetate:propionate, mol/mol	1.6	1.6	0.12	0.97

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect.

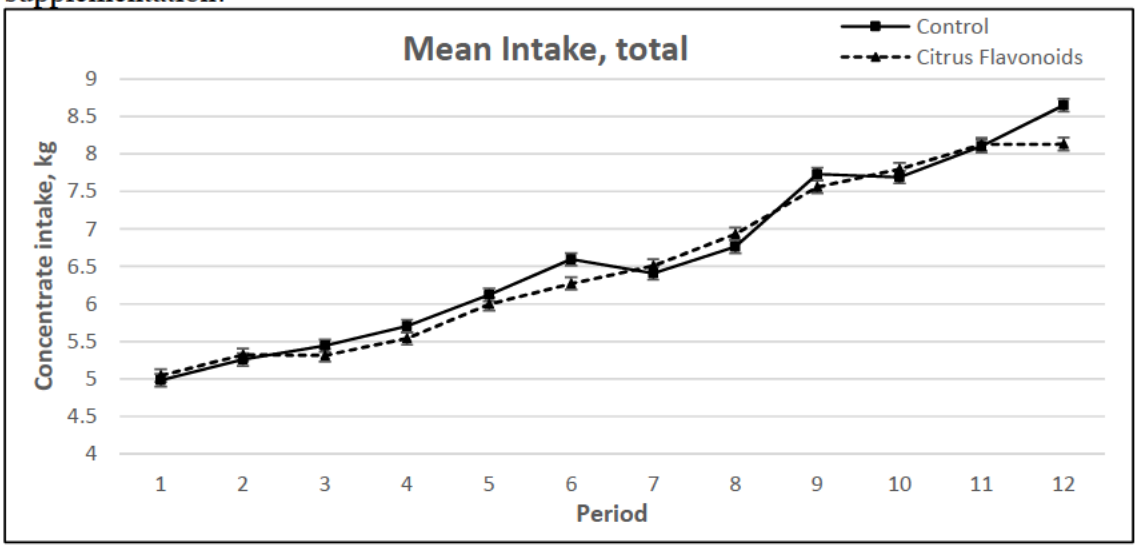
4. Discussion

In this study, the supplementation with citrus flavonoids throughout the fattening period did not affect performance parameters like ADG, final BW and FCR of bulls.

An interaction between treatment and time was observed throughout the study for concentrate intake. The **Figure 1** represents the mean of the concentrate intake by period throughout this study. Only in 2 periods a statistical decrease in concentrate intake was observed in BF bulls compared with C bulls. From period 7 to the end of the study an

erratic behavior for concentrate intake (greater CV) was observed, especially for C bulls. In this study, bulls were around 7 months old between period 6 and 7 of the study, moment that coincides with the unset of the puberty when an increase of production and secretion of testosterone occurs (Amann and Walker, 1983; Kenny and Byrne, 2018). **Figures 2** and **3** illustrate flehmen and complete mounts by period, respectively. Regarding flehmen behavior (**Figure 2**), C bulls clearly and steady increased the number of flehmen behaviors preformed from period 7 till the end of the study, whilst BF bulls' flehmen behaviors remained more stables. Furthermore, C bulls also exhibited higher number of complete mounts than BF bulls from period 7 until period 12 (**Figure 3**). How this modulation of sexual behaviors by citrus flavonoid supplementation occurs and if this behavior modulation is related with the more erratic concentrate intake observed in C bulls after puberty is not known.

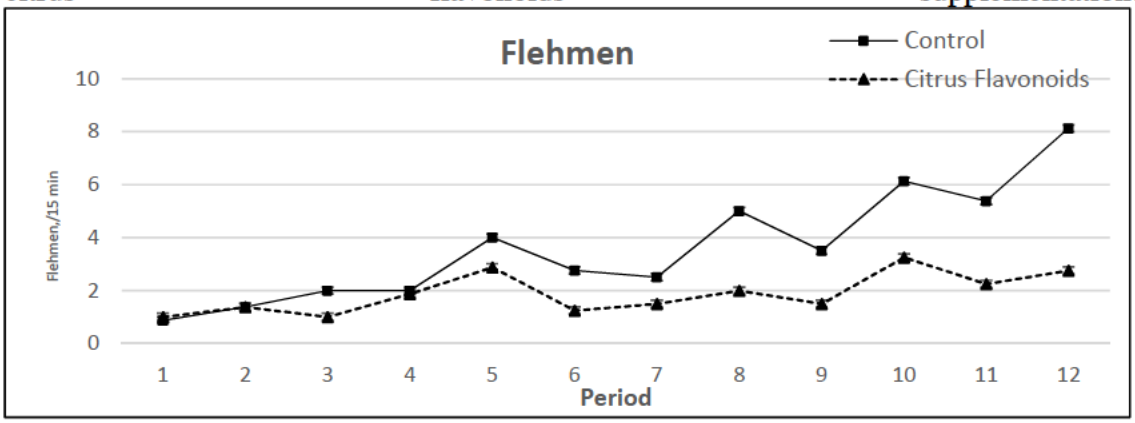
Figure 1. Mean of the concentrate intake during the growing and finishing phase of Holstein bulls fed high-concentrate diets with or without citrus flavonoids supplementation.

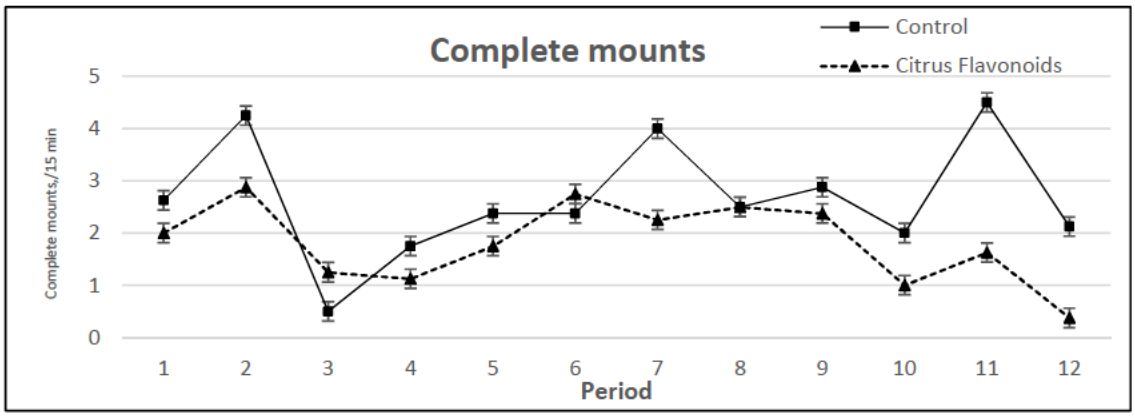


However, overall concentrate intake in the present study did not differ among treatments. In a previous study, carried out with a single space feeder in order to study the eating pattern of Holstein bulls fed high-concentrate diets supplemented with flavonoids (Chapter III), a reduction in the percentage of large meal sizes along with a reduction in concentrate intake were observed, that probably negatively affected final BW of bulls. Consequently, the use of a multi-space feeder in the present study would have

allowed to bulls supplemented with flavonoids to increase the number of visits to the feeder, allowing them to increase feed intake, even if percentage of large meal sizes was reduced and animals needed more time to eat, as observed in Chapter III. That would be supported by the visual scan procedure, as BF bulls occupied more time the concentrate feeder than C bulls during the growing phase. Although during the finishing phase this difference was only numerical, the percentage of BF bulls eating concentrate was also greater compared with C bulls. Interestingly, throughout this study BF bulls did not devoted more time to eat straw and no differences among treatments have been found for straw consumption. Conversely, higher occupancy of the straw feeder was observed in bulls supplemented with citrus flavonoids when a single-space feeder was used, so it could be hypothesized that in our previous study (Chapter III) BF bulls would have redirected their behavior to eat straw when they had not possibility to access to the concentrate feeder.

Figure 2 and 3. Flehmen and Complete mounts every 15 minutes during the growing and finishing phase of Holstein bulls fed high-concentrate diets with or without citrus flavonoids supplementation.





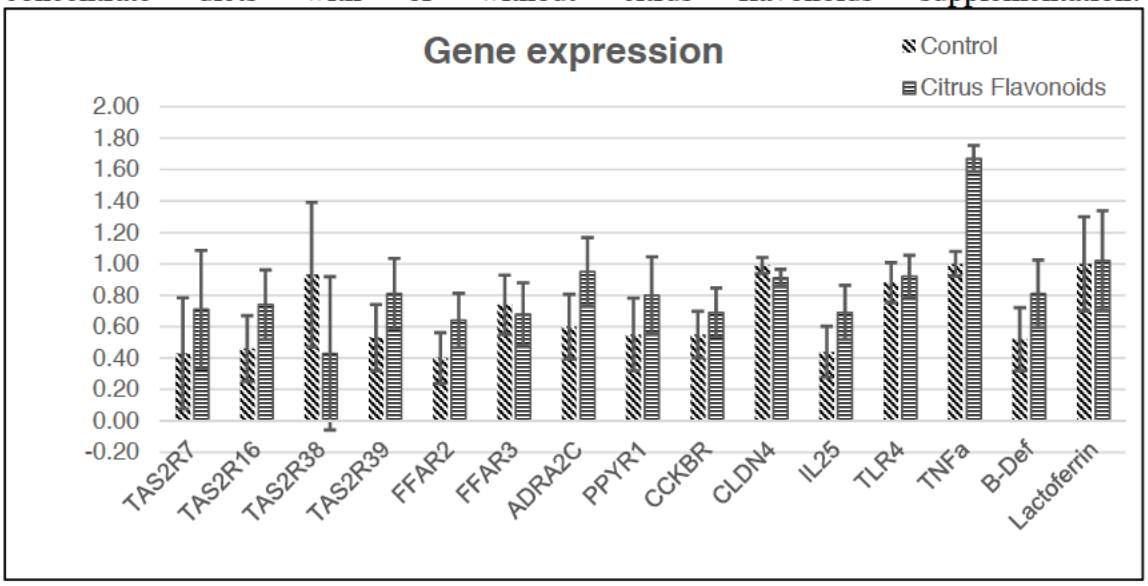
In previous studies *in vitro* and *in vivo*, an increase in molar proportions of propionic acid in the rumen was observed when citrus flavonoids were supplemented (Balcells et al., 2012; Seradj et al., 2014). As propionic acid has been described as key regulator of feed intake in ruminants fed high starch diets (Bradford and Allen, 2007), in Chapter III, it was hypothesized that propionic acid could be involve in the reduction of large meal sizes observed in bulls when citrus flavonoids were supplemented to the concentrate. In this study, ruminal fermentation parameters analyzed just after slaughtering did not evidence any difference among treatments, so propionate, acetate and total VFA were not affected. Also pH was similar among treatments. Regardless these results, differences among sampling method could affect rumen fluid VFA concentration and profile (Lam et al., 2018), so *in vitro* studies, in-farm (cannulated animals) sampling, or slaughter sampling probably could not be compared. Furthermore, accordingly with previous results (Chapter III), in the present study the color of the rumen wall was lighter for BF bulls. That would be related with better rumen wall health, and this could affect VFA absorption and, consequently, rumen concentration of VFA. On the other hand, the relative gene expression in ruminal epithelium of *ffar2* and *ffar3* are consistent with the results obtained for VFA in ruminal liquid, as well as no differences were observed among treatments for these nutrient sensing receptors (Hudson et al., 2012; Friedrichs, 2015).

As mentioned previously, another possible mode of action of citrus flavonoids to regulate eating behavior could be related with taste receptors (chemosensory transduction). Taste is a large sensory system responsible for food selection, but also in charge of detection of nutrients, minerals and toxins, being also vital for nutrient absorption regulation and energy homeostasis (Takay et al., 2016). Basic tastes include sweet, bitter, umami, soar and salty, and were initially described in taste buds of the tongue and different tissues of the mouth, although recently a great number of studies have described the presence of these taste receptors throughout different organs and systems (Behrens and Meyerhof, 2011). Bitter taste receptors (*TAS2R*) belong to the G protein-coupled receptors family (Behrens and Meyerhof, 2013; Lu et al 2017), and possess the important function of detecting heterogeneous chemical molecules that could be toxic (Favreau et al., 2010; Ginane et al., 2011). The extra-oral *TAS2R* would accomplish diverse functions, as innate immune response regulation in the respiratory system or secretion of different gut hormones in the gastrointestinal tract (Lu et al., 2017). Focusing on the digestive tract, these taste receptors would regulate gut hormones and

neurotransmitters release, and also nutrients uptake, being involved in hunger and satiety regulation (Depoortere, 2013). In fact, bitter chemicals would activate the release of different anorexigenic hormones and peptides in the gastrointestinal tract, such as ghrelin (orexigenic), cholecystokinin (*cck*), neuropeptide Y (*npy*), and peptide YY (*pyy*) (Chen et al., 2006; Depoortere, 2013; Takai et al., 2016).

Citrus flavonoids, and especially naringin, are responsible for the typical bitter taste of citrus fruits, so being able to activate these *TAS2R* (Drewnowski et al., 1997; Roland et al., 2014). Consequently, in this study different *TAS2R* were analyzed. The available literature is mainly based on human taste perception, so the *TAS2R* were chosen mainly based on this information. Human *TAS2R7* is activated by caffeine (Meyerhof et al., 2010; Poole and Tordof, 2017), *TAS2R16* is activated mainly by similar molecules than *TAS2R39*, both by dietary compounds and flavonoids from many different plant sources (Roland et al., 2014), depending if they are glycosylated or not (Meyerhof et al., 2010), and *TAS2R38* agonists are natural bitter molecules as well (Meyerhof et al., 2010; Ahmed et al., 2016). In the present study, only *TAS2R16* were affected by treatment, increasing the relative gene expression in BF bulls. This *TAS2R16* has been described as the bitter receptor for the phytonutrient β-glucopyranosides, which are very ubiquitous in nature (Bufe et al., 2002; Ji et al., 2014). In fact, this higher gene expression of *TAS2R16* in BF bulls might be related to naringin content of the citrus flavonoid extract supplemented, as naringin is a glycosylated flavanone (Tripoli et al., 2007). Furthermore, our results have also showed higher gene expression for *ppyr1* in bulls supplemented with flavonoids, which acts as *npy* and *pyy* receptor (Larhammar, 1996). As previously mentioned, *npy* and *pyy* are peptides released by *TAS2R* after a bitter stimuli, and whereas *pyy* is considered an anorexigenic hormone, *npy* has been reported as a collateral inhibitor for sweet taste cells when bitter taste cells are stimulated in taste buds (Depoortere, 2013; Takai et al., 2016). Although deeper research is needed, that could be related with the reduction in meal size observed in bulls when citrus flavonoids were supplemented in the concentrate and the reduced feed intake observed in BF bulls in the present study in some periods. Actually, possible functions of these *TAS2R* in rumen epithelium have not been studied, but with our results it could be hypothesize that citrus flavonoids would be acting over some *TAS2R* expressed in the rumen epithelium of bulls, modifying the release peptides involved in hunger and satiety and, consequently modulating eating pattern of these animals.

Figure 4. Gene expression in rumen epithelium of Holstein bulls fed high-concentrate diets with or without citrus flavonoids supplementation.



TAS2R7: *Bitter taste receptor 7;* TAS2R16: *Bitter taste receptor 16;* TAS2R38: *Bitter taste receptor 38;* TAS2R39: *Bitter taste receptor 39;* FFAR2: *Free fatty acid receptor 3 (gpr41);* FFAR3: *Free fatty acid receptor 2 (gpr43);* ADRA2C: *Alpha 2-adrenergic receptors subtype C;* PPYR1: *Pancreatic polypeptide receptor 1;* CCKBR: *Cholecystokinin receptor 4;* CLDN4*: Claudin4;* IL-25: I*nterleukin-25;* TLR4: *Pattern recognition receptors, like Toll-like receptor 4;* TNFa: *Tumor necrosis factor alpha;* B-Def: *Beta-defensin*. The values presented herein correspond to back-transformed means; however, SEM correspond to the ANOVA analyses using log-transformed data.

On the other hand, in agreement with our previous results (Chapter III), citrus flavonoid supplementation modified animal behavior in bulls. Animal welfare is an important and growing concern in modern societies, affecting consumers' opinion about livestock production systems, and consequently also the policies and regulations of intensive animal production. Moreover, animal well-being is related with a better health status and productivity, as chronic stressors impairing welfare are also associated with negative effects over the immune system of the animals (Carroll et al. 2007; Burdick et al., 2011). Therefore, it exists nowadays a great interest of all stakeholders, including farmers, to improve animal well-being, health and productivity. Animal behavior is considered as a measurement of animal welfare, and aggressive and oral non-nutritive behaviors have been described as important indicators of frustration and poor welfare in bulls (Gonyou et al., 1994; Bergeron et al., 2006). Recently, gut-brain axis has been suggested to be involved in animal behavior modulation, basically through inflammation and diet (Haagensen et al., 2014). The gut-brain axis is a communication network involved in the maintenance of gastrointestinal homeostasis, conveying information between digestive system and its microbiota with the brain (Haagensen et al., 2014;

Carabotti et al., 2015; Wiley et al., 2017). In beef cattle, some of these gut-brain crosstalk mechanisms affecting animal behavior have been proposed to take place in the rumen (Devant et al., 2016).

In the present study, bulls supplemented with citrus flavonoids reduced aggressive behaviors throughout the fattening period, and especially during the finishing phase sexual and oral non-nutritive behaviors were lesser performed by BF bulls. These results would be in agreement with our previous study (Chapter III), when bulls supplemented with the same citrus flavonoids extract also reduced oral non-nutritive behaviors, and aggressive and sexual interactions. Oral non-nutritive behaviors has been described as an abnormal oral behavior in ungulates, and digestive dysfunctions or little time devoted to chewing or ruminating behavior have been proposed as possible causes of this stereotypic behavior (Bergeron et al., 2006). In the present study, BF bulls performed less oral non-nutritive behaviors during the finishing phase, when they also exhibited greater ruminating activity than C bulls during the visual scan. Additionally, rumen wall color was lighter for bulls supplemented with citrus flavonoids, although pH at the slaughterhouse was not affected by treatment. Thus, it could be hypothesized that supplementation with citrus flavonoids reduced oral non-nutritive behaviors increasing ruminating activity and also improving rumen health. The mechanisms whereby citrus flavonoids modulate ruminating activity during this finishing phase, when BF bulls did not devoted more time to eat concentrate or straw remain unknown, and more research would be needed to elucidate them.

In agreement with our previous study (Chapter III), agonistic interactions studied and flehmen were reduced in BF bulls throughout the study, and attempted to mount behaviors during the finishing phase. Flavonoids supplementation might modulate animal behavior through mechanisms involved in the gut-brain crosstalk, and inflammation might be a key player. In the present study, citrus flavonoid supplementation increased the relative gene expression of molecules related with inflammation in rumen epithelium, such as *TNFa*, cytokine *IL-25* and *β-defensin*. Contrary to expected, inflammation could be involved in a decrease of serum serotonin concentrations, a neurotransmitter associated with mood modulation (Evans et al., 2013) and a reduction in aggressive behaviors (Haagensen et al., 2014). On the other hand, naringin could act as an antioxidant molecule (Harborne and Williams, 2000), explaining lighter color of the rumen wall in BF bulls.

Consequently, these results would be in contradiction, being necessary further investigations to discern if citrus flavonoids supplementation might modulate animal aggressive and sexual behaviors through rumen inflammation mechanisms, or if this modulation take place beyond the rumen, probably in the intestine as suggested for monogastric species.

Otherwise, as proposed in our previous study (Chapter III), eating and animal (social and sexual) behaviors would be interrelated, and increasing time devoted to eat might reduce aggressive and abnormal behaviors in animals (Qaisrarni et al., 2012). In the present study, when bulls were supplemented with citrus flavonoids dedicated more time to eat concentrate or performed greater ruminating activity, so that could lead to a reduction in aggressive and sexual interactions observed during the visual scan procedure. Thus, we could hypothesized that citrus flavonoids supplementation reduced agonistic and sexual interactions by increasing time devoted to perform eating behaviors, as ruminating or eating concentrate.

5. Conclusions

In conclusion, supplementation with flavonoids extracted from *Citrus aurantium* in bulls fed high-concentrate diets in pellet form reduced agonistic interactions and oral non-nutritive behaviors. Moreover, flavonoid supplementation modified the expression of genes in the rumen epithelium that could be related with inflammation and nutrient sensing, but further research would be needed to fully understand how flavonoids are modulating eating and animal behavior in bulls.

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CHAPTER V

Citrus aurantium flavonoid extract improves concentrate efficiency, animal behavior, and reduces rumen inflammation of Holstein bulls fed high-concentrate diets

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ABSTRACT

One hundred fourty-four bulls (164.8 ± 5.91 kg BW and 135 ± 7.2 d of age) were randomly allocated to one of 8 pens and assigned to control (C) or citrus flavonoid (BF) treatments (*Citrus aurantium*, 0.4 kg per ton of concentrate of Bioflavex CA, > 20% naringin; BF). Each pen had one drinker, one separate five-space straw feeder, and one separate three-space feeder where mash concentrate containing mostly corn, barley, DDG and wheat was offered. Concentrate intake was recorded daily, whilst BW and animal behavior were recorded fortnightly. Animals were slaughtered after 168 d of study (12 periods of 14 d), and HCW and carcass quality were recorded, and rumen papillae samples were collected. Data were analyzed using a mixed-effects model with repeated measures and categorical data with a Chi-Square. Final BW (437.9 ± 1.85 kg), HCW (238.7 ± 2.02 kg), and concentrate intake (7.1 ± 0.13 kg/d) were not affected by treatment. Concentrate feed conversion ratio (kg of concentrate/ kg of BW) tended ($P < 0.10$) to be lesser in BF (5.11 ± 0.108 kg/kg) than in C (5.36 ± 0.108 kg/kg) bulls. Percentage of animals eating concentrate during visual scan was greater ($P < 0.01$) in BF (10.02 % ± 0.512) compared with C (7.97 % ± 0.512). Oral non-nutritive behaviors, agonistic interactions (fighting, butting, and chasing) and sexual behaviors (flehmen, attempted and complete mounts) were greater ($P < 0.01$) in C than in BF bulls. In the rumen epithelium, gene expression of *bitter taste receptor 7*, *bitter taste receptor 16*, *bitter taste receptor 38* and *bitter taste receptor 39* was greater ($P < 0.05$) in C compared with BF bulls, as well as was gene expression of *free fatty acid receptor 2*, *pancreatic polypeptide receptor 1*, *cholecystokinin receptor 4*, *cytokine IL-25*, *Toll-like receptor-4* and *β-defensin1*. In conclusion, supplementation with flavonoids extracted from *Citrus aurantium* in bulls fed high-concentrate diets tends to improve efficiency, and reduces oral non-nutritive behaviors, agonistic interactions and sexual behavior. Moreover, flavonoid supplementation modifies the expression of genes in the rumen epithelium that could be related with eating and animal behavior regulation.

Keywords: bulls, flavonoids, performance, behavior, rumen inflammation, bitter taste receptors.

1. Introduction

Phytochemicals are chemical substances found in vegetables and edible fruits. They play important functions in plants (Martinez et al., 2017), acting as protecting molecules from harmful agents (insects, bacteria) or stressful situations (UV, temperature, lack of water). Otherwise, phytochemicals have showed biological activities and healthy effects in humans (Middleton et al., 2000) and animals (Tipu et al., 2006; Tripoli et al., 2007; Hong et al., 2012). Flavonoids are polyphenols that have been deeply studied, and *Citrus* fruits are considered the major source of flavonoids, containing a wide range of these phytochemicals. Recently, the effect of an extract from bitter orange (*Citrus aurantium*) rich in naringin has been studied in Holstein bulls fed high-concentrate diet during the growing and finishing phase (Chapter III). Bulls supplemented with citrus flavonoid extract modified their eating pattern, by reducing large meal sizes (>750 g/ meal) and spending more time eating straw, and rumen wall health parameters analyzed were improved. However, final BW and carcass weight were numerically reduced in bulls supplemented with citrus flavonoids. As this study was conducted with single-space feeders to register eating pattern of the bulls, this might modify total daily feeder access compared with commercial situations where feeders have multiple spaces (Verdú et al., 2015). It was hypothesized that such impact was the result of the use of single-space feeders, which were limiting the total daily access of the animals to the concentrate supplemented with citrus flavonoids, limiting also the potential maximum daily concentrate intake, and therefore performance (final BW and carcass). Consequently, in the present study citrus flavonoid extract supplementation will be tested in a commercial farm with multiple-space feeders.

Supplementation with citrus flavonoids reduced agonistic behaviors throughout the fattening period, and sexual interactions during the finishing phase in past studies (Chapter III and IV). The mechanisms whereby citrus flavonoids may modulate eating and animal behavior are unknown. Previously, studies with other extracts containing also naringin exhibited beneficial effects in regulating rumen pH, modulating ruminal microflora and ruminal fermentation (Balcells et al., 2012). This modulation of ruminal fermentation by citrus flavonoids affected volatile fatty acids (VFA) production in the rumen, increasing molar proportions of propionic acid (Balcells et al., 2012; Seradj et al., 2014), which is involved in the regulation of feed intake in ruminants feed high-starch

diets by stimulating satiety (Oba and Allen, 2003; Bradford and Allen, 2007). Moreover, naringin is the flavonoid responsible of the typical bitter taste in some citrus fruits (Ribeiro et al., 2008). Bitter taste is one of the five basic tastes (sweet, salty, bitter, soar and umami) perceived by humans and animals (Jaggupilli et al., 2016), and has been often considered as a negative value (Favreau et al., 2010). Recent studies have demonstrated that taste receptors, including bitter taste receptors (*TAS2R*), are expressed in the gastrointestinal tract (Behrens and Meyerhof, 2011; Breer et al., 2012). Thus, *TAS2R* could be involved in eating pattern modulation of bulls observed when concentrate was supplemented with citrus flavonoids. Finally, it has been suggested that inflammation, microbiota, and diet may affect animal behavior (Haagensen et al., 2014) by the gut-brain axis crosstalk. Devant et al. (2016) studied the gene expression of receptors involved in crosstalk mechanisms of the gut-brain axis in Holstein bulls fed different diets and suggested that some of these gut-brain crosstalk mechanisms could take place in the rumen.

The present study was designed to evaluate the effects of citrus flavonoid extract supplementation on concentrate consumption, growth rate, feed conversion ratio, macroscopic rumen wall health, carcass characteristics, and eating and animal behavior of Holstein bulls fed high-concentrate diets in commercial conditions with a multi-space feeder. Furthermore, the present study also aimed to investigate more deeply how citrus flavonoids supplementation could affect the expression of some genes in the rumen epithelium involved in gut-brain crosstalk mechanisms, such as taste receptors and inflammation regulators, that could explain differences related to the eating pattern and animal behavior.

2. Materials and methods

2.1. Animals, feeding, housing, and experimental design

This study was conducted in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado, 2013). One hundred fourty-four Holstein bulls (164.8 ± 5.91 kg of BW and 135 ± 7.2 d of age) were fattened under commercial conditions in a farm (Granja l'Alsina,

L'Alsina, Lleida). The whole study lasted 168 d, and was divided into growing (0 to 112 d) and finishing (113 to 168 d) phase. Animals were randomly allocated in one of eight 8 pens, and assigned to one of the two treatments (4 pens per treatment and 18 animals/ pen), either control (C) or supplemented (BF) with 0.04 % of bitter orange extract (*Citrus aurantium*) of the whole fruit rich in naringin (>20%) (Bioflavex CA, Interquim, S.A., Barcelona, Spain). Bioflavex was incorporated into the concentrate during the concentrate manufacturing. Concentrates were manufactured from a 9,000 kg master-batch, of which 4,500 kg were C, and the other 4,500 kg BF. Each treatment concentrate was transported to the farm with the same truck, and stored into two different silos under the same conditions.

Pens were totally covered (12 m x 6 m) and were deep-bedded with straw and equipped with a three-space feeder (1.50 m length, 0.40 m width, 1.50 m height, and 0.35 m depth). The feeder of each pen weighed the concentrate continuously as described by Verdú et al. (2017), and these data were recorded to calculate concentrate consumption by pen. Pens were also equipped with one drinker (0.30 m length, 0.30 m width, 0.18 m depth). Straw was offered *ad libitum* in a separated straw five-space feeder (3.60 m length, 1.10 m wide, and 0.32 m depth), and every time it was replaced it was recorded to estimate the total straw consumption. As straw was also used for bedding, these data are only an estimation.

2.2. Feed consumption and performance

Animals were fed a commercial concentrate in meal form, formulated to cover their nutritional requirements (FEDNA, 2008). The first 112 d of the study, animals were fed a grower concentrate formula, and between 112 d to the end of the study, animals were fed a finisher concentrate. Ingredients and nutritional composition of the concentrates are showed in **Table 1**. Throughout the study, animals had *ad libitum* access to wheat straw (3.5 % CP, 1.6 % ether extract, 70.9 % NDF, and 6.1 % ash; DM basis) and fresh water.

Table 1. Ingredients and nutrient composition of the feed concentrates.

Item	Growing[1]	Finishing[2]
Ingredient, g/ kg		
Corn grain meal	399.7	450.9
Barley grain meal	179.8	155.5
DDGs	179.8	150.2
Wheat	109.7	110.3
Beet pulp	73.9	80.2
Palm oil	20.0	25.0
Calcium carbonate	15.5	12.8
Urea	8.0	4.0
Sodium bicarbonate	5.0	4.0
Dicalcium phosphate	3.6	3.1
Vitamin premix	3.0	2.0
Salt	2.0	2.0
Nutrient		
ME, Mcal/kg DM	3.30	2.97
CP, g/ kg DM	157	123
Ether extract, g/ kg DM	58	54
Ash, g/ kg DM	56	44
NFD, g/ kg DM	178	151
NFC, g/ kg DM	550	628

[1] from 0 to 112 days of the study.
[2] from 113 days to the end of the study.

Animals were weighed individually every 14 d throughout the study in 12 experimental periods of 14 d. As already mentioned, during the 8 first periods (from 1 d to 112 d) the animals consumed the growing concentrate and during the last 4 periods (from 113 d to 168 d) and during the days before slaughter animals consumed the finishing concentrate (see Table 1). After 168 d of study bulls were transported to the slaughterhouse (Escorxador del Grup Alimentari Guissona, Guissona, Spain), located 15 km from the farm. Animals were slaughtered in two weeks, 4 pens per week, two pens from C and two from BF bulls each week. The time waiting before slaughter was less than 6 h. Animals were weighed before loading. They were slaughtered by commercial practices and following the EU Regulation 1099/2009 on the protection of animals at the time of killing or slaughtering.

2.3. Animal behavior

A visual scan procedure at days 15, 30, 43, 57, 71, 85, 94, 112, 127, 141, 155, and 170 of the study was performed to study the general activity (standing, lying, eating, drinking, and ruminating) and social behavior (nonagonistic, agonistic, and sexual interactions) of the animals in every pen. Social behavior activities recorded are described in **Table 2**, **Chapter III**.

General activities recorded were: consumption (when an animal had its head into the feeder and was engaged in chewing) of concentrate, and straw, drinking (when an animal had its mouth in the water bowl), ruminating (including regurgitation, mastication and swallowing of the bolus). Also, postures such as standing or lying (sternal recumbence with all legs folded under the body with the head down or up) were recorded. The visual observation was made for 2 pens at the same time from 8:00 to 10:30 h am, as described by Verdú et al. (2015). General activities were scored using 3 scan samplings of 10 s at 5 min intervals, and social behavior was scored during three continuous sampling periods of 5 min. This scanning procedure of 15 min was repeated twice consecutively in each pen, starting randomly in a different pen every scanning day. This method describes a behavior exhibited by an animal at a fixed time interval (Colgan, 1978).

2.4. Carcass quality

After slaughtering, HCW was registered for every animal. Dressing percentage was calculated by dividing HCW by BW recorded before slaughtering. And, following the (S)EUROP categories described by the EU Regulation No. 1208/81 and 1026/91, conformation of carcasses was classified, where "E" corresponded to an excellent conformation, "U" to very good conformation, "R" to good conformation, "O" to fair conformation, and "P" to a poor conformation. The fat cover was classified according the EU Regulation No. 1208/81, which utilizes a classification system by numbers, 1.2.3.4.5, where 5 explains a very high degree of covering fat and heavy fat deposits in the thoracic cavity, and 1 is classified as low degree, with no fat cover.

2.5. Rumen and liver macroscopic evaluation and sample collection

Rumen and liver of every animal were macroscopically evaluated at the slaughterhouse. Rumens were classified depending on the color by a visual evaluation, from 1 to 5, being "5" a black colored rumen and "1" a white colored rumen (González et al., 2001). They were also divided into areas according to Lesmeister et al. (2004) to examine the presence of ulcers, baldness regions, and of clumped papillae (Nocek et al., 1984). Liver abscesses were classified according to Brown et al. (1975).

Additionally, a liquid sample from rumen was obtained from homogeneous contents strained with a cheesecloth from 18 animals randomly selected from two pens per treatment, immediately following slaughter. Following the procedures of Jouany (1982), 4 mL of ruminal fluid was mixed with 1 mL of a solution containing 0.2% (wt/wt) mercuric chloride, 2% (wt/wt) orthophosphoric acid, and 2 mg/mL of 4-methylvaleric acid (internal standard) in distilled water, and stored at -20ºC until subsequent VFA analysis. Also, a 1-cm2 section of rumen wall (left side of the cranial ventral sac) was sampled and papillae were excised before rinsed 2 times with chilled PBS after sampling and immediately incubated in RNA-later (Invitrogen, Madrid, Spain) to preserve the RNA integrity. After 24 hours of incubation with RNA later at 4 ºC, the liquid was removed and tissue was frozen at -80 ºC until further RNA extraction and subsequent gene expression analysis.

2.6. Biological and chemical analyses

During the study, samples of concentrate were collected at d 0, 42, 84, 126, and 168 d. and analyzed for DM (method 925.04; AOAC, 2005), ash (method 642.05; AOAC, 2005), CP by the Kjeldahl method (method 988.05; AOAC, 2005), ADF and NDF according to Van Soest et al. (1991) using sodium sulfite and alpha-amylase, and EE by Soxhlet with a previous acid hydrolysis (method 920.39; AOAC, 2005).

Naringin was determined for every sample of concentrate (C and BF) as a Bioflavex CA marker for BF group, and was used as a marker confirming adequate inclusion of citrus flavonoid extract in the diets by Laboratory of Interquim S.A. Internal method for naringin quantification using HLPC developed by Interquim S.A. was used (Chapter III).

Ruminal VFA concentration was determined with a semicapillary column (15 m × 0.53 mm ID, 0.5 µm film thickness, TRB-FFAP, Teknokroma, Barcelona, Spain) composed of 100% polyethylene glycol (PEG) esterified with nitroterephtalic acid, bonded and crosslinked phase (method number 5560*;* APHA–AWWA–WPCF, 2005), using a CP-3800-GC (Varian, Inc., Walnut Creek, CA).

For gene expression analyses, total RNA was extracted from rumen wall homogenizing tissues in Trizol (Invitrogen) by Polytron Instrument (IKA, Germany). Isolated mRNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (Takara, Frankfurt, Germany) following the manufacturer's instructions. The RNA purity was assessed by a NanoDrop instrument (ThermoFisher, Madrid, Spain) at 260, 280, and 230 nm. The quantification of the expression of genes at the mRNA level coding for 1) the tight-junction protein Claudin4 *(CLDN4)*; 2) the production, expression, and turnover of neurotransmitters: free fatty acid receptor 2 (*ffr2*) and free fatty acid receptor 3 (*ffr3*), pancreatic polypeptide receptor 1 (*ppyr1)*; actual name neuropeptide Y receptor Y4 [*npy4r*]), and α2-adrenergic receptor subtype C (*adra2c*), cholecystokinin receptor 4 (*cckbr*); 3) pro-inflammatory cytokines TNF-α (*TNFα*) and cytokine IL-25 *(IL-25)*, pattern recognition receptor Toll-like receptor 4 *(TLR4)* and antimicrobial peptides released by intestinal cells (*β-defensins*, and *lactoferrin*); 4) bitter taste receptors type 2 member 7, 16 ,38 and 39 (*TAS2R7, TAS2R16, TAS2R38* and *TAS2R39)* were performed by quantitative PCR (qPCR). The qPCR was performed using gene codifying for Ribosomal Protein Subunit 9 (*RPS9*) as a housekeeping gene, which was checked for stability following Vandesompele et al. (2002) in comparison with genes codifying for β-actin (*ACTB*), ubiquitously expressed Transcript protein (*UXT*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The qPCR conditions for each set of primers were individually optimized (**Table 2, Chapter IV**). The specificity of the amplification was evaluated by single band identification at the expected molecular weight in 0.8% DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by amplifying serial 1:10 dilutions of each gene amplicon. A standard curve of crossing point (Cp) versus the logarithm of the concentration was plotted to obtain the efficiency, which was calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8 to 2.2. A total reaction volume of 20 µL was used, containing 50 ng of cDNA, 10 µL of SYBR Premix EXTaq (TliRNAseH) (Takara, Frankfurt, Germany) and the optimized primer concentration for each gene (**Table 2, Chapter IV**). The qPCR reactions were performed

as follows: an initial denaturing step of 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C, and a final extension of 10 min at 72°C. The resulting Cp values were used to calculate the relative expression of selected genes by relative quantification using a reference gene (housekeeping gene) and a calibrator of control group (Pfaffl, 2004, Eq. [3.5]).

2.7. Calculations and statistical analyses

Only pen was considered the experimental unit and animals within pen were considered sampling units in some parameters.

Concentrate efficiency data were transformed into log to achieve a normal distribution. The means presented in the tables and figures correspond to non-transformed data and, SEM and P-values correspond to the ANOVA analyses of the transformed data. The percentage of each general activity was calculated, and the average by day, pen, and scan obtained. Then, these data were transformed into natural logarithms to achieve a normal distribution. The frequency of each social behavior was calculated by summing by day, pen, and scan, and transformed into the root of the sum of each activity plus 1 to achieve a normal distribution. The ANOVA analysis was performed with transformed data, and the means shown in the tables correspond to the back transformed data.

Unification of performance, animal behavior and concentrate consumption data averaged by pen and period were analyzed using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC). The model included initial BW as a covariate, treatment, period (14-d period), and the interaction between treatment and period, as fixed effects, and the interaction between treatment and pen and the 3-way interaction between treatment, pen, and period as random effects. Period was considered a repeated factor, and for each analyzed variable, animal nested within the interaction between treatment and pen (the error term) was subjected to 3 variance-covariance structures: compound symmetry, autoregressive order one, and unstructured. The covariance structure that yielded the smallest Schwarz's Bayesian information criterion was considered the most desirable analysis.

In the case of rumen gene expression and VFA data pen were considered the experimental unit and animals as sampling units, and data were analyzed using ANOVA

where the model included treatment (as there were no repeated measures) as the main effect. For categorical variables analyses (carcass classification, rumen health parameters, hepatic abscesses) a Chi-square-test was used.

Differences were declared significant at $P < 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

3. Results

3.1. Animal health

Two animals from the C treatment were removed before the study end due to lameness problems. One animal from the BF treatment was also removed due to chronic respiratory problems.

Table 2. Performance and concentrate intake for growing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	134.25	135.22	0.215	<0.001		
Final age, d	246.16	247.22	0.689	0.32		
Initial BW, kg	165.03	164.64	5.906	0.96		
Final BW (168 d of study), kg	360.34	360.27	1.282	0.97		
CV, %	8.65	9.37	0.773	0.54	<.0001	0.34
ADG, kg/d	1.75	1.75	0.011	0.97	<.0001	0.58
CV, %	19.98	22.15	0.934	0.11	0.0011	0.29
Concentrate DM intake						
Mean, kg/d	6.83	6.60	0.143	0.26	<.0001	0.46
CV, %	10.53	11.29	0.700	0.45	0.0002	0.95
FCR, kg/kg	4.50	4.34	0.087	0.19	<.0001	0.22

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

3.2. Intake, performance and carcass quality

No statistical differences were found in concentrate intake between treatments, neither during the growing phase (**Table 2**) nor for the finishing phase (**Table 3**). Also for the whole study (**Table 4**), no differences between treatments were observe for this parameter. In the same way, estimated straw consumption did not show statistical differences during the growing phase ($P = 0.92$) (0.57 ± 0.046 kg/d and 0.56 ± 0.046 kg/d

for C and BF, respectively) nor for the finishing phase (P = 0.46) (0. 89 ± 0.074 kg/d and 0.97 ± 0.074 kg/d for C and BF, respectively) (data not presented). Throughout the study straw consumption estimated were 0. 73 ± 0.080 kg/d for C group and 0.77 ± 0.080 kg/d for BF animals, and no statistical differences were observed either (P = 0.74) (data not presented).

Table 3. Performance and concentrate intake for finishing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	246.16	247.22	0.689	0.32		
Final age, d	302.02	303.22	0.677	0.25		
Initial BW, kg	360.34	360.27	1.212	0.97		
Final BW (168 d of study), kg	436.34	439.48	1.849	0.28		
CV, %	7.04	8.24	0.308	0.01	0.82	0.76
ADG, kg/d	1.36	1.41	0.019	0.048	<.0001	0.35
CV, %	42.83	36.28	1.831	0.02	0.018	0.95
Concentrate DM intake						
Mean, kg/d	7.96	7.91	0.193	0.86	0.022	0.39
CV, %	11.68	11.43	0.767	0.82	0.63	0.70
FCR, kg/kg	7.08	6.66	0.297	0.34	<.0001	<0.05

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

During the growing phase the ADG was not affected by treatment. However, ADG during the finishing phase was greater (P < 0.05) for BF bulls than for C bulls, and CV of ADG was lesser (P < 0.05) for BF bulls compared with C bulls. Otherwise, CV of final BW was greater (P = 0.01) for BF bulls than for C bulls. Concentrate FCR tended (P = 0.10) to be less for BF bulls than for C bulls at the end of the 168 d of study, although BW and concentrate intake were not affected by treatment. Furthermore, CV of water intake was greater (P < 0.05) for C bulls than for BF bulls during the finishing phase. An interaction between treatment and time was found for concentrate FCR (P < 0.05) thorough the study and during the finishing phase without any clear pattern.

Carcass quality data are presented in **Table 5**. At the slaughterhouse BW, dressing percentage, carcass conformation and fatness classification were not affected by treatment.

Table 4. Performance and concentrate intake for the whole study in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	134.25	135.22	0.215	<0.001		
Final age, d	302.02	303.22	0.677	0.25		
Initial BW, kg	165.03	164.64	5.906	0.96		
Final BW (168 d of study), kg	436.34	439.48	1.849	0.28		
CV, %	8.10	8.91	0.764	0.46	<.0001	0.34
ADG, kg/d	1.62	1.64	0.011	0.19	<.0001	0.57
CV, %	27.60	26.85	1.564	0.74	<.0001	0.61
Concentrate DM intake						
Mean, kg/d	7.21	7.04	0.126	0.37	<.0001	0.51
CV, %	10.93	11.32	0.623	0.66	0.0002	0.97
FCR, kg/kg	5.36	5.11	0.108	0.10	<.0001	0.03

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

Table 5. Carcass quality from Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P-value*[2]
Item	C	BF	SEM	T
Age before slaughter, d	313.38	314.75	0.916	0.29
Days in study, d	179.37	179.53	0.431	0.80
BW before slaughter, kg	450.39	452.62	3.154	0.62
Hot carcass weight, kg	237.60	239.92	2.019	0.42
Dressing percentage, %	52.80	53.07	0.326	0.56
Fatness, %				-
1				
2				
3	100	100		
Conformation, %				0.37
P	91.43	84.93		
O	8.57	13.70		
R	0	1.37		
U				

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect.

3.3. Animal behavior

All data for animal behavior, general activities and active behavior as well, are showed in **Table 6** and **Table 7** for growing and finishing phase, respectively.

General activities. No statistical differences were found in the percentage of animals per pen standing, lying, eating straw and ruminating throughout the visual observation period for the growing phase. The percentage of animals eating concentrate was greater ($P < 0.01$) for BF compared C bulls, and the proportion of animals drinking water tended ($P < 0.10$) to be greater as well for BF bulls than for C bulls during this phase.

For the finishing phase, no differences were found in the proportion of animals per pen standing, lying, eating straw and drinking water during the visual observation period. In this phase, the proportion of animals per pen eating concentrate tended ($P < 0.10$) to be greater in BF bulls compared with C bulls, and the proportion of animals ruminating in BF group was also greater ($P < 0.01$) than for C bulls.

Active behavior. In the growing phase, during the visual scan observation period, the only parameter not affected by treatment was the social behavior. Self-grooming behavior was greater for BF compared with C bulls, and C bulls exhibited more ($P < 0.01$) oral non-nutritive behaviors than BF bulls. BF bulls exhibited less ($P < 0.01$) agonistic interactions than C bulls. Fighting, displacement, chasing and chasing-up, and butting behaviors were greater ($P < 0.05$) in C than in BF bulls. Flehmen behavior was greater ($P < 0.05$) in C compared with BF bulls. Additionally, attempt to mount and complete mounts tended ($P < 0.10$) to be greater in C than BF bulls.

During the finishing phase no differences between treatments were observed for social and oral behaviors. Bulls from the C group tended ($P < 0.10$) to perform more self-grooming behaviors than BF bulls. Otherwise, differences among treatments in agonistic and sexual behaviors became more evident; C bulls exhibited more agonistic and sexual behaviors than BF bulls.

Table 6. General activities (%) and social behavior (times/ 15 min) for growing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
General Activity, %						
Standing	55.64	58.80	0.100	0.60	<0.0001	0.99
Lying	44.36	41.15	0.115	0.58	<0.001	0.99
Eating concentrate	8.95	11.07	0.040	<0.0001	<0.0001	0.82
Eating straw	4.87	7.45	0.059	0.35	0.188	0.44
Drinking	1.40	2.59	0.015	0.09	0.06	0.89
Ruminating	12.24	14.74	0.128	0.70	<0.01	0.86
Social behavior, /15 min						
Selfgrooming	15.22	18.23	0.091	<0.01	<0.0001	<0.05
Social	4.61	5.97	0.181	0.14	<0.0001	0.99
Oral non-nutritive	1.41	0.88	0.078	<0.01	0.177	0.78
Fighting	7.42	3.20	0.147	<0.001	<0.001	0.91
Butting	4.19	1.61	0.091	<0.0001	<0.0001	0.73
Displacement	1.69	1.09	0.038	<0.001	<0.001	0.85
Chasing	0.84	0.30	0.063	<0.05	0.140	0.70
Chasing up	0.16	0.02	0.027	<0.05	0.103	0.22
Flehmen	3.03	2.00	0.148	<0.05	<0.05	0.63
Attempt to mount	1.76	0.75	0.101	0.09	<0.0001	0.31
Complete mounts	1.33	0.91	0.098	0.09	<0.0001	0.31

1. C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.

2. T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.

3. SEM = standard error of the means of the log-transformed data (general activity) or root transformed data (social behavior).

Table 7. General activities (%) and social behavior (times/ 15 min) for finishing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
General Activity, %						
Standing	63.46	64.11	0.041	0.51	0.739	0.70
Lying	35.44	35.68	0.122	0.75	0.881	0.86
Eating concentrate	6.00	7.91	0.059	0.09	0.395	0.65
Eating straw	4.27	6.25	0.068	0.24	0.072	0.69
Drinking	1.87	1.93	0.	0.87	0.203	0.85
Ruminating	7.33	12.68	0.049	<0.0001	0.194	0.99
Social behavior, /15 min						
Selfgrooming	8.62	7.00	0.122	0.08	0.480	0.80
Social	4.69	3.53	0.222	0.24	0.451	0.92
Oral non-nutritive	0.94	0.38	0.108	0.20	0.271	0.54
Fighting	10.34	3.53	0.288	<0.0001	<0.001	0.49
Butting	4.63	2.00	0.224	<0.001	0.046	0.50
Displacement	0.78	0.28	0.089	<0.001	0.143	0.91
Chasing	1.62	0.19	0.051	<0.0001	<0.0001	<0.05
Chasing up	0.13	0.03	0.016	0.12	0.450	0.17
Flehmen	4.91	3.78	0.182	0.07	<0.01	0.86
Attempt to mount	8.01	2.32	0.324	<0.001	<0.0001	<0.05
Complete mounts	6.16	2.52	0.252	<0.001	<0.0001	0.20

1. C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
2. T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.
3. SEM = standard error of the means of the log-transformed data (general activity) or root transformed data (social behavior).

3.4. Macroscopic rumen evaluation and liver abscesses

At the slaughterhouse (**Table 8**), color of rumen wall was lighter (P < 0.05) for BF bulls (63.01% classified as color < "3") compared with C bulls (45.71 classified as color < "3"). Baldness areas presence in the rumen were greater (P < 0.05) in BF bulls (58.90%) than in C bulls (38.57%). No differences between treatments were observed in the remaining macroscopic parameters analyzed at the slaughterhouse (liver abscesses, ulcers and clumped papillae).

Table 8. Macroscopical observations of the rumen and liver at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]		*P*-value[2]
Item	C	BF	
Color of the rumen[3]			< 0.05
> 3	54.29	36.99	
< 3	45.71	63.01	
Papillae clumping			0.74
Yes	22.86	20.55	
No	77.14	79.45	
Baldness region			< 0.05
Yes	38.57	58.90	
No	61.43	41.10	
Liver abscess[4]			0.51
None	87.14	83.56	
A	4.29	2.74	
A-	2.86	8.22	
A+	1.43	0	
Inflammation	4.29	5.48	

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect.
[3]Adapted from Gonzalez et al. (2001): Rumen color: 1= white; 5 = black.
[4]Adapted from Nocek et al. (1984).

3.5. Rumen VFA concentration at slaughterhouse

Rumen VFA concentration data are presented in **Table 9**. Total VFA concentration in the rumen was not affected by treatment. The molar proportion of acetate was greater ($P < 0.001$) in BF bulls compared with C bulls, whereas molar proportion of propionate was greater ($P < 0.001$) for C bulls than for BF bulls. Accordingly, acetate:propionate ratio was greater ($P < 0.001$) for the BF bulls than for C bulls. The remaining of VFA analyzed (butyrate, valerate, isobutyrate and isovalerate) were not affected by the treatment.

Table 9. Rumen VFA concentration at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P-value*[2]
	C	BF	SEM	
Rumen				
Total VFA, m*M*	75.6	67.2	4.76	0.22
Individual VFA, mol/100 mol				
Acetate	58.8	66.4	1.12	<0.0001
Propionate	28.9	20.7	1.21	<0.0001
Isobutyrate	7.4	7.5	0.34	0.90
n-butyrate	1.2	1.5	1.13	0.11
IsoValerate	1.5	1.3	0.13	0.26
Valerate	2.1	2.5	0.24	0.22
Acetate:propionate, mol/mol	2.15	3.35	0.171	<0.0001

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect.

3.6. Expression of genes in the rumen epithelium

The relative expression at mRNA level of genes studied in the rumen epithelium are presented in **Figure 1**. The supplementation with flavonoids affected the expression of all the bitter taste receptors (*TAS2R*) analyzed. The relative expression of *TAS2R7*, *TAS2R16*, *TAS2R38*, and *TAS2R38* were greater ($P < 0.01$) in the rumen of C compared with BF bulls. The relative expression of receptors related with the neurotransmitter signaling differ among treatment. The *ffar3* ($P = 0.10$) and *ffar2* ($P < 0.01$) were greater expressed in this C bulls compared with BF bulls. In addition, the relative expression for *ppyr1* and *cckbr* was greater ($P < 0.01$) as well for C bulls than for BF bulls. Furthermore,

the relative expression of the receptors related with the inflammation like *IL-25, TLR4*, and *defensin*, were greater ($P < 0.05$) for C bulls than for BF bulls.

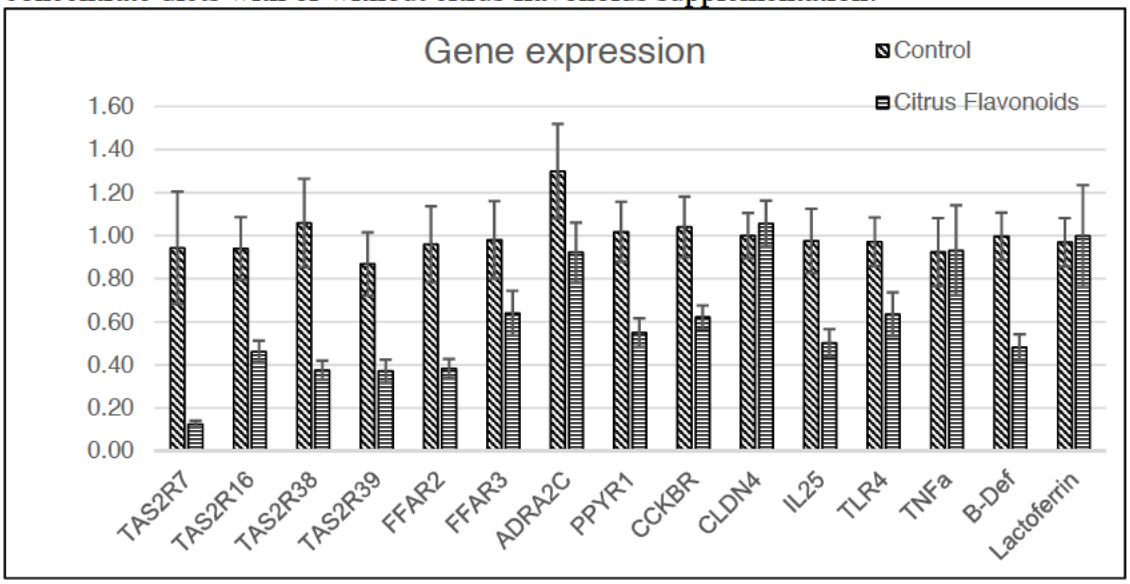
4. Discussion

In this study, flavonoid supplementation tended to improve feed efficiency of bulls. Previously (Chapter III), a reduction in concentrate intake explained by a numerical decrease of meal size was found in bulls supplemented with flavonoids fed with a single-space feeder. Consequently, the hypothesis that the single-space feeder could have been limiting the access to the concentrate was considered, especially during the finishing phase, when bulls possibly were not able to compensate the decrease of meal size by increasing the number of visits to the feeder. Therefore, in the current study, multiple-space feeders were used and, during the finishing phase, bulls supplemented with flavonoids did not exhibit a reduction of the concentrate intake. Moreover, during this phase, concentrate feeder occupancy tended to be greater for BF animals. This supports the hypothesis that these animals were able to compensate meal size reduction by increasing the number of visits to the feeder. Additionally, in the present study BF bulls showed higher ADG than C bulls during this finishing phase without an increase of concentrate intake. Consequently, BF bulls were more efficient during this phase, although concentrate efficiency improvement was only numerical. Smaller meal sizes have been related to an improvement of feed efficiency in steers (Montanholi et al., 2010). Then, assuming that bulls supplemented with flavonoids may have reduced the large meal sizes (> 750 g/ meal) (Chapter III), this could explain the improvement in concentrate efficiency during the finishing phase and the greater ADG of these BF animals. In a previous *in vitro* study Seradj et al. (2014) observed that BF decreased methane production which could explain the efficiency improvement in BF supplemented bulls observed in the present study. Another possible mechanism involved in the feed efficiency improvement in beef animals described in the literature is based on the rumen VFA profile. Greater propionate percentage and lesser acetate:propionate ratio in rumen fluid are related with better efficiency in ruminants when ionophore antibiotics are added to the feed (Golder and Lean, 2016). Previously, in the study carried out by Balcells et al. (2012), an increase in molar proportions of propionic acid in the rumen of cannulated heifers supplemented with citrus flavonoids was observed. Propionic acid has been

described as an important regulator of feed intake in ruminants fed high starch diets (Bradford and Allen, 2007). According to these results, the hypothesis that supplementation with citrus flavonoids could reduce large meal sizes in bulls due to an increase in ruminal propionate production was proposed (Chapter III). However, the results of the current study showed greater rumen molar percentage of propionate at slaughterhouse in C bulls than in animals supplemented with flavonoids. Furthermore, rumen molar percentage of acetate was greater for BF bulls and, consequently, acetate:propionate ratio was lower for C bulls compared with bulls supplemented with flavonoids. Sampling, in-farm or slaughter can affect rumen fluid VFA concentration and profile (Lam et al., 2018). Rumen epithelium health could affect VFA absorption. Accordingly with previous results (Chapter III), in the present study the color of the rumen wall was lighter for BF bulls, and this might be indicative of better rumen wall health. As propionate is rapidly absorbed by simple diffusion in the timeframe of the meal (Allen et al., 2009; Allen and Bradford, 2012), whilst for acetate absorption an active transport is needed (Aschenbach et al., 2014), a better health of the rumen epithelium could explain that BF bulls showed lesser rumen concentration of propionate content compared with C animals. Although BF bulls had greater baldness area in the rumen, and this could translated with a reduced capacity of absorption, this cannot be affirmed as total absorption surface of the rumen was not measured in the present study.

On the other hand, *ffar2* and *ffar3* tended to be lesser expressed in the rumen epithelium of BF compared with C bulls. These results would be coherent with the differences obtained in VFA profiles between BF and C bulls, as both nutrient sensing receptors, *ffar2* and *ffar3*, are greater stimulated by propionate compared with acetate in bovine (Hudson et al., 2012; Friedrichs, 2015). Although their functions are not well established in bovine, and further research is needed, these nutrient sensing receptors are involved in the modulation of the release of several gastrointestinal hormones (Mielenz, 2017), modulating eating pattern as well. Furthermore, as discussed later in the behavior effects of BF supplementation, the expression of *ffar3* could be related with serotonin release (Evans et al., 2013). Serotonin is a monoamine neurotransmitter that is involved in the regulation of learning, mood, sleep, anxiety, and other psychiatry-related afflictions and recently it has been studied as a signaling molecule linking the brain and the gut (Evans et al., 2013).

Figure 1. Gene expression in rumen epithelium of Holstein bulls fed high-concentrate diets with or without citrus flavonoids supplementation.



TAS2R7: *Bitter taste receptor 7;* TAS2R16: *Bitter taste receptor 16;* TAS2R38: *Bitter taste receptor 38;* TAS2R39: *Bitter taste receptor 39;* FFAR2: *Free fatty acid receptor 3 (gpr41);* FFAR3: *Free fatty acid receptor 2 (gpr43);* ADRA2C: *Alpha 2-adrenergic receptors subtype C;* PPYR1: *Pancreatic polypeptide receptor 1;* CCKBR: *Cholecystokinin receptor 4;* CLDN4*: Claudin4;* IL-25: I*nterleukin-25;* TLR4: *Pattern recognition receptors, like Toll-like receptor 4;* TNFa: *Tumor necrosis factor alpha;* B-Def: *Beta-defensin*.

Moreover, taste receptors were initially discovered in taste buds located in the tongue and different parts of the oral cavity. Recently, an important number of studies are describing the presence of taste receptors for the basic tastes (sweet, bitter, umami, soar and salty) throughout the body, including respiratory system and gastrointestinal tract (Behrens and Meyerhof, 2011). This peripheral gustatory system would have the function of tasting the luminal content of the digestive tract, and regulating nutrient transporters expression, nutrients uptake, and also the release of gut hormones and neurotransmitters involved in the regulation of the gastrointestinal function, feeding and satiety (Depoortere, 2013). Bitter molecules trigger the release of mainly anorexigenic hormones and peptides, such as *ghrelin* (orexigenic), *cholecystokinin* (*cck*), *neuropeptide Y* (*npy*), and *peptide YY* (*pyy*) (Chen et al., 2006; Depoortere, 2013; Takai et al., 2016). This would be a logical response, as bitter taste has been often related to the presence of toxins (Favreau et al., 2010; Ginane et al., 2011), and is considered to have a negative value (Favreau et al., 2010). Thus, the activation of an anorexigenic response in the digestive tract would be an adaptive response to this taste. The *TAS2R* analyzed were chosen mainly based on human literature. Human *TAS2R39* agonists are dietary compounds and flavonoids from many different plant sources (Roland et al., 2014), whereas *TAS2R7* is

activated by caffeine (Meyerhof et al., 2010; Poole and Tordof, 2017). *TAS2R16* is activated mainly by similar molecules than *TAS2R39*, depending if they are glycosylated or not (Meyerhof et al., 2010), and *TAS2R38* agonists are natural molecules as well, and this bitter taste receptor has been related to the immune system in humans (Meyerhof et al., 2010; Ahmed et al., 2016). In the present study, flavonoids supplementation reduced the gene expression of all *TAS2R* analyzed in the rumen epithelium (*TAS2R7*, *TAS2R16*, *TAS2R38*, and *TAS2R39)*. Although naringin has a characteristic bitter taste, it is rapidly deglycosylated by enzymes to naringenin (Busto et al., 2007), and rumen microflora is able to degrade naringin to naringenin (Simpson et al., 1969; Cheng et al., 1971) as well. Contrary to naringin, naringenin acts as an important bitter masking molecule (Jacob et al., 2014). Roland et al. (2014) described how some flavanones act as actual antagonists for human *TAS2R39*, reducing the receptor response possibly by orthosteric mechanism acting over a single binding pocket of the bitter taste receptor. Accordingly, our results would agree with the function of naringenin as a bitter masking molecule, acting as antagonist for all *TAS2R* analyzed. This reduction in the gene expression of *TAS2R* could be related with the greater time devoted to eat observed in BF bulls. Actually, our results have also showed a clear decline in the gene expression of receptors related with neurotransmitters, as *cckbr* (acts as *cck* and *gastrin* receptor; Silvente-Poirot and Wank, 1996) and *ppyr1* (acts as *npy* and *pyy* receptor; Larhammar, 1996) in bulls supplemented with flavonoids, that would be in concordance with the reduction in the expression of the *TAS2R* analyzed in the rumen epithelium of these animals, because of these taste receptors are involved in the release of these anorexigenic molecules. Consequently, we can hypothesize that supplementation with citrus flavonoids in bulls might modulate eating pattern acting over *TAS2R* expressed in the rumen epithelium, and consequently modifying the release of hormones and bioactive peptides involved in hunger and satiety. Therefore, some citrus flavonoids or their derivates might act as bitter masking molecules in the rumen, and bulls supplemented with these flavonoids devoted more time to eat, and this could be related to a decrease in agonist and sexual interactions.

Although reducing meal size and increasing occupancy of the feeder at the same time could be considered a contradiction, in fact, naringin supplemented in the concentrate is a bitter molecule that would be triggering the release of anorexigenic hormones and peptides, until is metabolized into naringenin. Conversely, naringenin would be acting as a bitter masking molecule, reducing the release of these anorexigenic

hormones and activating eating behavior. However, more research is needed to deeply investigate the interrelationships between naringin, naringenin and how both flavonoids act over the eating behavior in bulls. Moreover, the effects of flavonoids or their derivates on rumen fermentation may affect other nutrients like amino acids or VFA that may affect the receptors related with the nutrients sensing mechanisms that alter eating pattern.

Flavonoid supplementation affected animal behavior. Thus, it is important to have in mind that nowadays animal welfare is considered an important issue in animal production systems, and in developed societies there is a growing concern about wellbeing of farm animals. Consequenly, improving animal welfare is a challenge that intensive animal production systems will have to face in the coming years. Nutritive strategies (Devant et al., 2016; Celi et al., 2017), the use of different feed additives (McGrath et al., 2018), and enriching the environment in the farms (Casal-Plana et al., 2017) have been proposed as possible alternatives to ameliorate animal welfare. In beef cattle, some animal behaviors, as aggressive and oral non-nutritive behaviors, have been described as indicators of poor welfare, frustration and discomfort (Gonyou et al., 1994; Devant et al., 2016). Gut-brain axis has been proposed as a communication network between digestive system and brain, and may affect behavior in humans and other animals (Haagensen et al., 2014; Devant et al., 2016; Wiley et al., 2017). Beyond the mechanisms described above related to the nutrient sensing mechanisms, Devant et al. (2016) suggested that the rumen could be involved in the crosstalk between digestive system and brain in beef cattle, indicating that animal aggressive and sexual behaviors could be modulated by this axis.

Previously, in bulls supplemented with the same citrus flavonoids used in this study, a reduction of oral non-nutritive behaviors, and aggressive and sexual interactions were observed (Chapter III and IV). In agreement with these previous results, in the present study BF bulls exhibited less oral non-nutritive behaviors during the growing phase. Licking objects with non-nutritional finality has been described as an abnormal oral behavior in cattle, and digestive dysfunctions, as rumen lesions, low rumination activity and low pH, have been proposed as possible causes of this behavior (Bergeron et al., 2006; Devant et al., 2016). Furthermore, in the present study, ruminating activity was greater in BF bulls during the finishing phase, and rumen wall color was lighter for BF bulls. Thus, it could be hypothesized that supplementation with citrus flavonoids reduced

oral non-nutritive behaviors modulating eating pattern of bulls, and improving rumen health.

As previously observed (Chapter III and IV), in the present study all agonistic and sexual interactions studied were reduced in BF bulls compared with non-supplemented animals throughout the study. Mechanisms whereby flavonoids supplementation may reduce these agonistic and sexual behaviors are unknown. Flavonoids supplementation might modulate animal behavior through mechanisms involved in the gut-brain crosstalk. Some of those mechanisms could be related with nutrient sensing mechanisms (capacity to sense and respond to nutrients) being here bitter taste and *ffar* receptors key players. On the other hand, molecules regulating gastrointestinal inflammation and neuropeptides could also have a relevant involvement in animal behavior modulation.

In the present study, citrus flavonoids supplementation has clearly reduced the gene expression of the proteins related with the inflammation in the rumen epithelium of the bulls such as cytokine *IL-25, TLR4*, and *β-defensin*. Inflammation has been suggested to play an important role in animal welfare (Haagensen et al., 2014; Wiley et al., 2017) and behavior (Devant et al., 2016), possibly by the gut-brain axis crosstalk. Inflammation could be involved in a decrease of serum serotonin concentrations, a neurotransmitter that plays an important role within the gut-brain axis, and has been associated with mood modulation (Evans et al., 2013) and a reduction in aggressive behaviors (Haagensen et al., 2014). Additionally, selective serotonin reuptake inhibitors (which increase extracellular serotonin) have been related to libido reduction and sexual problems in humans (Balon, 2006). Cytokine *IL-25* is produced by a variety of cells, including immune and non-immune cells (epithelial and endothelial) and it can potentiate allergic inflammation (Gu et al., 2013). *TLR4* is involved in the recognition of endotoxin of gram-negative bacteria and LPS (Yunhe et al., 2013), and plays a fundamental role in pathogen recognition and activation of innate immunity (Lu et al., 2008). *B-defensin* is an antimicrobial peptide, and have modulatory effects on innate and adaptive immune processes in mammals (Yang et al., 2001 and 2007). Thus, in our study, citrus flavonoids supplementation reduced the gene expression of these inflammation-related molecules, and this could be leading to a reduction in inflammatory response and inflammation in the rumen epithelium. Furthermore, naringenin could act as a potent antioxidant and its anti-inflammatory effects has been deeply described (Manchope et al., 2017). According

to the previous results (Chapter III and IV), in the present study aggressive and sexual interactions in bulls supplemented with citrus flavonoids were reduced, and rumen gene expression data would support that the reduction of the rumen inflammation could be a key player in this response.

Finally, as mentioned before when analyzing eating and animal (social and sexual) behaviors one should have in mind that they could be interrelated. Qaisrarni et al. (2012) described that some nutritional strategies, focused on increasing time devoted to eat, reduced aggressive and abnormal behaviors of the animals. In this case, bulls supplemented with flavonoids dedicated more time to eat concentrate or ruminating during the visual scan procedure. Thus, we could not reject the hypothesis that animals devoting more time to feeding events had less time to perform other behaviors, as aggressive and sexual interactions.

5. Conclusions

In conclusion, concentrate intake regulation (time devote to eat) together with the inflammation and other gut-brain crosstalk mechanisms in the rumen epithelium might be involved in the improvement of animal behavior and welfare along with efficiency of bulls supplemented with citrus flavonoids fed high-concentrate diets.

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CHAPTER VI

Citrus aurantium flavonoid extract in high-fat finishing diets improves animal behavior, rumen health and modifies the gene expression in rumen and duodenum epithelium of Holstein bulls

DRAFT

ABSTRACT

One hundred fourty-six bulls (178.2 ± 6.64 kg BW and 146.0 ± 0.60 d of age) were randomly allocated to one of 8 pens and assigned to control (C) or (BF) (*Citrus aurantium*, Bioflavex CA, Interquim S.L., Spain, 0.4 kg per ton of concentrate of Bioflavex CA, 24% naringin). Each pen had one drinker, one separate five-space straw feeder, and one separate three-space feeder where mash concentrate rich in corn, barley, DDG and wheat was offered. At the finishing phase, up to 350 kg of BW, fat content of the concentrate was increased from 58 to 84 g/kg DM by increasing palm oil inclusion rate. Concentrate intake was recorded daily, and BW and animal behavior by visual scan, fortnightly. Animals were slaughtered after 168 d of study (12 periods of 14 d), HCW and carcass quality were recorded, and rumen and duodenum epithelium samples were collected. Final BW (440.3 ± 9.95 kg), overall concentrate intake (6.4 ± 0.15 kg/d) and overall concentrate FCR (4.13 ± 0.117 kg/kg) were not affected by treatment. However, in the growing phase concentrate intake were greater (P<0.05) for C bulls (6.1 ± 0.12 kg/d) compared with BF bulls (5.8 ± 0.12 kg/d). During the visual scan procedure, percentage of animals eating concentrate and ruminating were greater (P < 0.05) throughout the study in BF compared with C bulls, whereas during the growing phase percentage of bulls eating straw was also greater (P < 0.01) in BF than in C bulls. At slaughterhouse, ruminal pH was greater (P < 0.01) in BF bulls compared with C bulls, whilst total VFA content was greater (P < 0.01) in C than in BF bulls. The molar proportion of acetate was greater (P < 0.01) in BF compared with C bulls. In contrast, molar proportion of propionate was greater (P < 0.01) for C than for BF bulls, so acetate:propionate ratio was greater (P < 0.05) in BF bulls as well. Oral non-nutritive behaviors, agonistic interactions (fighting, butting, and chasing) and flehmen were greater (P < 0.01) exhibited in C than in BF bulls. Attempted mounts were greater (P = 0.01) performed by C compared with BF bulls during the growing phase, whereas sexual behaviors (attempted and complete mounts) in the finishing phase tended (P < 0.10) to be also greater performed in C than in BF bulls. In the rumen epithelium, gene expression of *bitter taste receptor 7, bitter taste receptor 16,* and *bitter taste receptor 39* was greater (P < 0.05) in BF compared with C bulls, as well as gene expression of *free fatty acid receptor 2, free fatty acid receptor 3, α2-adrenergic receptor subtype C, pancreatic polypeptide receptor 1, cholecystokinin receptor 4, cytokine IL-25, Toll-like receptor-4* and *β-defensin*. Gene expression of *Claudin4* tended (P < 0.10) to be greater in rumen

epithelium of C compared with BF bulls. Conversely, in duodenum epithelium the majority of these genes were less ($P < 0.05$) expressed in BF than in C bulls. In conclusion, citrus flavonoids supplementation reduced concentrate intake in bulls during the growing phase. Additionally, citrus flavonoids reduced oral non-nutritive behaviors, agonistic interactions and sexual behaviors throughout the study. Ruminal pH and rumen wall parameters macroscopically analyzed at slaughterhouse suggested better rumen health in BF than in C bulls. Moreover, flavonoid supplementation differently modified the gene expression in the rumen and duodenum epithelium when high-fat concentrate was fed, and this could be related with eating pattern and animal behavior regulation.

Keywords: bulls, flavonoids, performance, behavior, rumen inflammation, bitter taste receptors.

1. Introduction

Citrus fruits contain a wide range of flavonoids. These flavonoids are polyphenols, a category of phytochemicals plenty of biological activities, such as anti-inflammatory, antioxidant, and antimicrobial properties (Harborne and Williams, 2000). In previous research carried out with an extract from bitter orange (*Citrus aurantium*) rich in naringin (Bioflavex CA, Interquim, S.A., Spain), citrus flavonoids supplementation modified the eating pattern of Holstein bulls fed high-concentrate diets throughout the fattening period, and reduced the large meal sizes performed by the animals during the finishing phase (Chapter III). Furthermore, bulls supplemented with citrus flavonoids devoted more time to eat concentrate, straw and performed more ruminating activity during the finishing phase (Chapter III, IV and V). Moreover, when the concentrate was fed in meal form, although final BW and concentrate intake were not affected by treatment, feed conversion ratio tended to improve in bulls supplemented with these citrus flavonoids (Chapter V). Additionally, flavonoids supplementation reduced the gene expression of all the bitter taste receptors (*TAS2R*) studied in ruminal epithelium when concentrate was fed in meal form (Chapter V) but, on the contrary, the gene expression of some of these genes was increased when concentrate was fed in pellet form (Chapter IV). The expression of these different genes could be related to gut-brain axis mechanisms (Chapter IV, V). Consequently, nutrient-sensing and other gut-brain crosstalk mechanisms in the rumen wall might be involved in the modulation of animal eating pattern and behavior along with the improvement of efficiency in bulls supplemented with citrus flavonoids fed high-concentrate diets in meal form.

Different nutritional strategies allow to modulate eating pattern and intake in cattle, as increasing fat content in the feed (Heinrichs et al., 1982; Devant et al., 2013; Martí et al., 2014). Furthermore, an increase in blood concentration of cholecystokinin (*cck*) and pancreatic polypeptide (*pp*) has been related to the reduction in feed intake associated with high fat levels in the diet of cows (Choi and Palmquist, 1996). Actually, *pp* is considered a member of the *npy* family, composed neuropeptide Y (*npy*), peptide YY (*pyy*) and *pp*, acting all of them upon the same family of receptors, *npyr* (Larhammar, 1996; Michel et al., 1998). Bulls supplemented with flavonoids in concentrate fed in meal form have showed a decline in the gene expression of receptors related with these neurotransmitters and hormones, as *cckbr* (acts as *cck* and gastrin receptor; Silvente-

Poirot and Wank, 1996) and *ppyr1* (Chapter V). In commercial conditions, high fat diets have been used to modulate feed intake and reduce the meal size in cattle after the prohibition of ionophore antibiotics (monensin) in Europe, mainly to control bloat and rumen acidosis problems. Thus, effects observed in cattle when high fat diets are used might be similar than these observed when concentrate has been supplemented with citrus flavonoids in our studies, also reducing meal size and concentrate intake. Additionally, in both cases (fat and flavonoids), metabolic pathways involving *cck* and *npy* family are likely playing a key role. Consequently, the possible existence of an interaction between fat level and citrus flavonoid supplementation in high-concentrate diets should be studied under commercial conditions in beef cattle.

Finally, oral non-nutritive behaviors, agonistic interactions (fighting, butting, and chasing) and sexual behaviors (flehmen, attempted and complete mounts) were also reduced in bulls supplemented with flavonoids, independently of the feeder space and the concentrate presentation (pellet or meal) (Chapter III, IV and V). Our previous studies showed that flavonoid supplementation modulated the expression of some genes related to the gut-brain axis in the rumen of bulls, although these results have been affected by presentation form of the concentrate, pellet (Chapter IV) or meal (Chapter V). Thus, high-fat concentrate could be also affecting the expression of these genes when citrus flavonoids are supplemented.

Thus, the present study was designed to evaluate the effects of citrus flavonoid supplementation on concentrate consumption, growth rate, concentrate efficiency, macroscopic rumen wall health, carcass characteristics, and animal behavior in Holstein bulls fed high-fat concentrate diets in commercial conditions. Furthermore, the present study also aimed to investigate deeper how citrus flavonoid supplementation of high fat concentrate diets could affect the expression of some genes involved in gut-brain crosstalk mechanisms in the rumen and duodenum epithelium, such as bitter taste receptors and inflammation regulators.

2. Materials and methods

2.1. Animals, feeding, housing, and experimental design

This study was conducted in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado, 2013). One hundred fourty-four Holstein bulls (164.8 ± 5.91 kg of BW and 135 ± 7.2 d of age) were fattened under commercial conditions in a farm (Granja l'Alsina, L'Alsina, Lleida). The whole study lasted 168 d, and was divided into growing (0 to 112 d) and finishing (113 to 168 d) phase. Animals were randomly allocated in one of eight 8 pens, and assigned to one of the two treatments (4 pens per treatment and 18 animals/ pen), either control (C) or supplemented (BF) with 0.04 % of bitter orange extract (*Citrus aurantium*) of the whole fruit rich in naringin (>20%) (Bioflavex CA, Interquim, S.A., Barcelona, Spain). Bioflavex was incorporated into the concentrate during the concentrate manufacturing. Concentrates were manufactured from a 9,000 kg master-batch, of which 4,500 kg were C, and the other 4,500 kg BF. Each treatment concentrate was transported to the farm with the same truck, and stored into two different silos under the same conditions.

Pens were totally covered (12 m x 6 m) and were deep-bedded with straw and equipped with a three-space feeder (1.50 m length, 0.40 m width, 1.50 m height, and 0.35 m depth). The feeder of each pen weighed the concentrate continuously as described by Verdú et al. (2017), and these data were recorded to calculate concentrate consumption by pen. Pens were also equipped with one drinker (0.30 m length, 0.30 m width, 0.18 m depth). Straw was offered *ad libitum* in a separated straw five-space feeder (3.60 m length, 1.10 m wide, and 0.32 m depth), and every time it was replaced it was recorded to estimate the total straw consumption. As straw was also used for bedding, these data are only an estimation.

2.2. Feed consumption and performance

Animals were fed a commercial concentrate in meal form, formulated to cover their nutritional requirements (FEDNA, 2008). The first 112 d of the study, animals were fed a grower concentrate formula, and between 112 d to the end of the study, animals

were fed a finisher concentrate. Ingredients and nutritional composition of the concentrates are showed in **Table 1**. Throughout the study, animals had *ad libitum* access to wheat straw (3.5 % CP, 1.6 % ether extract, 70.9 % NDF, and 6.1 % ash; DM basis) and fresh water.

Table 1. Ingredients and nutrient composition of the feed concentrates.

Item	Growing[1]	Finishing[2]
Ingredient, g/ kg		
Corn grain meal	399.7	436.9
Barley grain meal	179.8	150.2
DDGs	179.8	150.2
Wheat	109.7	109.8
Beet pulp	73.9	80.0
Palm oil	20.0	45.0
Calcium carbonate	15.5	12.8
Urea	8.0	4.0
Sodium bicarbonate	5.0	4.0
Dicalcium phosphate	3.6	3.1
Vitamin premix	3.0	2.0
Salt	2.0	2.0
Nutrient		
ME, Mcal/kg DM	3.21	3.29
CP, g/ kg DM	157	136
Ether extract, g/ kg DM	58	84
Ash, g/ kg DM	56	46
NFD, g/ kg DM	178	169
NFC, g/ kg DM	551	565

[1] from 0 to 112 days of the study.
[2] from 113 days to the end of the study.

Animals were weighed individually every 14 d throughout the study in 12 experimental periods of 14 d. As already mentioned, during the 8 first periods (from 1 d to 112 d) the animals consumed the growing concentrate and during the last 4 periods (from 113 d to 168 d) and during the days before slaughter animals consumed the finishing concentrate (see **Table 1**). After 168 d of study bulls were transported to the slaughterhouse (Escorxador del Grup Alimentari Guissona, Guissona, Spain), located 15 km from the farm. Animals were slaughtered in two weeks, 4 pens per week, two pens

from C and two from BF bulls each week. The time waiting before slaughter was less than 6 h. Animals were weighed before loading. They were slaughtered by commercial practices and following the EU Regulation 1099/2009 on the protection of animals at the time of killing or slaughtering.

2.3. Animal behavior

A visual scan procedure at days 7, 23, 39, 49, 64, 78, 90, 106, 122, 137, and 147 of the study was performed to study the general activity (standing, lying, eating, drinking, and ruminating) and social behavior (nonagonistic, agonistic, and sexual interactions) of the animals in every pen. Social behavior activities recorded are described in **Table 2, Chapter III**.

General activities recorded were: consumption (when an animal had its head into the feeder and was engaged in chewing) of concentrate, and straw, drinking (when an animal had its mouth in the water bowl), ruminating (including regurgitation, mastication and swallowing of the bolus). Also, postures such as standing or lying (sternal recumbence with all legs folded under the body with the head down or up) were recorded. The visual observation was made for 2 pens at the same time from 8:00 to 10:30 h am, as described by Verdú et al. (2015). General activities were scored using 3 scan samplings of 10 s at 5 min intervals, and social behavior was scored during three continuous sampling periods of 5 min. This scanning procedure of 15 min was repeated twice consecutively in each pen, starting randomly in a different pen every scanning day. This method describes a behavior exhibited by an animal at a fixed time interval (Colgan, 1978).

2.4. Carcass quality

After slaughtering, HCW was registered for every animal. Dressing percentage was calculated by dividing HCW by BW recorded before slaughtering. And, following the (S)EUROP categories described by the EU Regulation No. 1208/81 and 1026/91, conformation of carcasses was classified, where "E" corresponded to an excellent conformation, "U" to very good conformation, "R" to good conformation, "O" to fair conformation, and "P" to a poor conformation. The fat cover was classified according the

EU Regulation No. 1208/81, which utilizes a classification system by numbers, 1.2.3.4.5, where 5 explains a very high degree of covering fat and heavy fat deposits in the thoracic cavity, and 1 is classified as low degree, with no fat cover.

2.5. Rumen and liver macroscopic evaluation and sample collection

Rumen and liver of every animal were macroscopically evaluated at the slaughterhouse. Rumens were classified depending on the color by a visual evaluation, from 1 to 5, being "5" a black colored rumen and "1" a white colored rumen (González et al., 2001). They were also divided into areas according to Lesmeister et al. (2004) to examine the presence of ulcers, baldness regions, and of clumped papillae (Nocek et al., 1984). Liver abscesses were classified according to Brown et al. (1975).

Additionally, a liquid sample from rumen was obtained from homogeneous contents strained with a cheesecloth from 18 animals randomly selected from two pens per treatment, immediately following slaughter. Following the procedures of Jouany (1982), 4 mL of ruminal fluid was mixed with 1 mL of a solution containing 0.2% (wt/wt) mercuric chloride, 2% (wt/wt) orthophosphoric acid, and 2 mg/mL of 4-methylvaleric acid (internal standard) in distilled water, and stored at -20ºC until subsequent VFA analysis. Also, a 1-cm2 section of rumen wall (left side of the cranial ventral sac) and duodenum epithelium were sampled. Ruminal papillae form the rumen wall section was excised, and both ruminal papillae and duodenum epithelium samples were rinsed 2 times with chilled PBS after sampling and immediately incubated in RNA-later (Invitrogen, Madrid, Spain) to preserve the RNA integrity. After 24 hours of incubation with RNA later at 4 ºC, the liquid was removed and tissue was frozen at -80 ºC until further RNA extraction and subsequent gene expression analysis.

2.6. Biological and chemical analyses

During the study, samples of concentrate were collected at d 0, 42, 84, 126, and 168 d. and analyzed for DM (method 925.04; AOAC, 2005), ash (method 642.05; AOAC, 2005), CP by the Kjeldahl method (method 988.05; AOAC, 2005), ADF and NDF according to Van Soest et al. (1991) using sodium sulfite and alpha-amylase, and EE by Soxhlet with a previous acid hydrolysis (method 920.39; AOAC, 2005).

Naringin was determined for every sample of concentrate (C and BF) as a Bioflavex CA marker for BF group, and was used as a marker confirming adequate inclusion of citrus flavonoid extract in the diets by Laboratory of Interquim S.A. Internal method for naringin quantification using HLPC developed by Interquim S.A. was used (Paniagua et al., 2018).

Ruminal VFA concentration was determined with a semicapillary column (15 m × 0.53 mm ID, 0.5 µm film thickness, TRB-FFAP, Teknokroma, Barcelona, Spain) composed of 100% polyethylene glycol (PEG) esterified with nitroterephtalic acid, bonded and crosslinked phase (method number 5560*;* APHA–AWWA–WPCF, 2005), using a CP-3800-GC (Varian, Inc., Walnut Creek, CA)**.**

For gene expression analyses, total RNA was extracted from ruminal and duodenum epithelium homogenizing tissues in Trizol (Invitrogen) by Polytron Instrument (IKA, Germany). Isolated mRNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (Takara, Frankfurt, Germany) following the manufacturer's instructions. The RNA purity was assessed by a NanoDrop instrument (ThermoFisher, Madrid, Spain) at 260, 280, and 230 nm. The quantification of the expression of genes at the mRNA level coding for 1) the tight-junction protein Claudin4 *(CLDN4)*; 2) the production, expression, and turnover of neurotransmitters: free fatty acid receptor 2 (*ffr2*) and free fatty acid receptor 3 (*ffr3*), pancreatic polypeptide receptor 1 (*ppyr1)*; actual name neuropeptide Y receptor Y4 [*npy4r*]), and α2-adrenergic receptor subtype C (*adra2c*), cholecystokinin receptor 4 (*cckbr*); 3) pro-inflammatory cytokines TNF-α (*TNFα*) and cytokine IL-25 *(IL-25),* pattern recognition receptor Toll-like receptor 4 *(TLR4)* and antimicrobial peptides released by intestinal cells (*β-defensins,* and *lactoferrin*); 4) bitter taste receptors type 2 member 7, 16 ,38 and 39 (*TAS2R7, TAS2R16, TAS2R38* and *TAS2R39)* were performed by quantitative PCR (qPCR). The qPCR was performed using gene codifying for Ribosomal Protein Subunit 9 (*RPS9*) as a housekeeping gene, which was checked for stability following Vandesompele et al. (2002) in comparison with genes codifying for β-actin (*ACTB*), ubiquitously expressed Transcript protein (*UXT*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The qPCR conditions for each set of primers were individually optimized (**Table 2, Chapter IV**). The specificity of the amplification was evaluated by single band identification at the expected molecular weight in 0.8% DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by

amplifying serial 1:10 dilutions of each gene amplicon. A standard curve of crossing point (Cp) versus the logarithm of the concentration was plotted to obtain the efficiency, which was calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8 to 2.2. A total reaction volume of 20 μL was used, containing 50 ng of cDNA, 10 μL of SYBR Premix EXTaq (TliRNAseH) (Takara, Frankfurt, Germany) and the optimized primer concentration for each gene (**Table 2, Chapter IV**). The qPCR reactions were performed as follows: an initial denaturing step of 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C, and a final extension of 10 min at 72°C. The resulting Cp values were used to calculate the relative expression of selected genes by relative quantification using a reference gene (housekeeping gene) and a calibrator of control group (Pfaffl, 2004, Eq. [3.5]).

2.7. Calculations and statistical analyses

Only pen was considered the experimental unit and animals within pen were considered sampling units in some parameters.

Concentrate efficiency data were transformed into log to achieve a normal distribution. The means presented in the tables and figures correspond to non-transformed data and, SEM and P-values correspond to the ANOVA analyses of the transformed data. The percentage of each general activity was calculated, and the average by day, pen, and scan obtained. Then, these data were transformed into natural logarithms to achieve a normal distribution. The frequency of each social behavior was calculated by summing by day, pen, and scan, and transformed into the root of the sum of each activity plus 1 to achieve a normal distribution. The ANOVA analysis was performed with transformed data, and the means shown in the tables correspond to the back transformed data.

Unification of performance, animal behavior and concentrate consumption data averaged by pen and period were analyzed using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC). The model included initial BW as a covariate, treatment, period (14-d period), and the interaction between treatment and period, as fixed effects, and the interaction between treatment and pen and the 3-way interaction between treatment, pen, and period as random effects. Period was considered a repeated factor, and for each analyzed variable, animal nested within the interaction between treatment

and pen (the error term) was subjected to 3 variance-covariance structures: compound symmetry, autoregressive order one, and unstructured. The covariance structure that yielded the smallest Schwarz's Bayesian information criterion was considered the most desirable analysis.

In the case of rumen gene expression data were transformed into log to achieve a normal distribution. The means presented in the figures correspond to non-transformed data and, SEM and P-values correspond to the ANOVA analyses of the transformed data. Pen were considered the experimental unit and animals as sampling units, and data were analyzed using ANOVA where the model included treatment (as there were no repeated measures) as the main effect. For VFA data, also pen were considered the experimental unit and animals as sampling units, and data were analyzed using ANOVA where the model included treatment (as there were no repeated measures) as the main effect. For categorical variables analyses (carcass classification, rumen health parameters, hepatic abscesses) a Chi-square-test was used.

Differences were declared significant at $P < 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

3. Results

3.1. Animal health

Two animals did not finish the study, one from C treatment due to enterotoxaemia and one form BF treatment due to chronic respiratory problems.

3.2. Intake, performance and carcass quality

During the growing phase (**Table 2**) concentrate intake was greater ($P < 0.05$) for C bulls than for BF bulls, whilst this differences disappeared for the finishing phase (**Table 3**). Furthermore, no statistical differences between treatments were found for this parameter when analyzing the whole study (**Table 3**). Moreover, the estimation for straw consumption did not show statistical differences between treatments during the growing phase ($P = 0.48$), being 0.54 ± 0.021 kg/d for C bulls and 0.52 ± 0.021 kg/d for BF

animals, and neither for the finishing phase (P = 0.21), when straw intake was 1.02 ± 0.132 kg/d and 0.76 ± 0.132 kg/d for C and BF bulls, respectively.

Table 2. Performance and concentrate intake for growing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	144.87	147.17	0.601	<0.05		
Final age, d	256.87	259.23	0.667	<0.05		
Initial BW, kg	178.17	178.13	6.638	0.99		
Final BW (112 d of study), kg	349.24	349.86	9.270	0.96		
CV, %	7.96	8.14	0.753	0.87		
ADG, kg/d						
Mean, kg/d	1.53	1.53	0.017	0.84	<.0001	0.11
CV, %	23.88	25.66	0.834	0.14	0.36	0.66
Concentrate DM intake						
Mean, kg/d	6.09	5.76	0.116	<0.05	<.0001	0.70
CV, %	14.49	15.23	0.935	0.58	<0.001	0.80
FCR, kg/kg	3.98	3.77	0.096	0.13	<.0001	0.26

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

Table 3. Performance and concentrate intake for finishing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	256.87	259.23	0.667	<0.05		
Final age, d	312.87	315.07	0.620	0.05		
Initial BW, kg	349.24	349.86	9.270	0.96		
Final BW (168 d of study), kg	439.65	440.95	9.953	0.93		
CV, %	8.01	8.13	0.558	0.89		
ADG, kg/d						
Mean, kg/d	1.62	1.63	0.034	0.81	0.35	0.27
CV, %	32.45	28.74	2.012	0.21	0.35	0.95
Concentrate DM intake						
Mean, kg/d	7.46	7.32	0.194	0.62	<.0001	0.50
CV, %	13.05	17.04	1.668	0.11	0.01	0.99
FCR, kg/kg	4.68	4.60	0.196	0.80	0.17	0.34

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

On the other hand, ADG, final BW and FCR, were not affected by treatment neither during the growing phase (**Table 2**) nor during the finishing phase (**Table 3**). Accordingly, these performance parameters also did not show statistical differences throughout the study between treatments (**Table 4**).

Table 4. Performance and concentrate intake for the whole study in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM	T	Time	T x Time
Initial age, d	144.87	147.17	0.601	<0.05		
Final age, d	312.87	315.07	0.620	<0.05		
Initial BW, kg	178.17	178.13	6.638	0.99		
Final BW (168 d of study), kg	439.65	440.95	9.953	0.93		
CV, %	8.01	8.13	0.558	0.89		
ADG, kg/d						
Mean, kg/d	1.56	1.56	0.018	0.98	<.0001	0.21
CV, %	26.75	26.69	0.850	0.96	<0.01	0.62
Concentrate DM intake						
Mean, kg/d	6.55	6.28	0.147	0.20	<.0001	0.63
CV, %	14.01	15.83	1.097	0.24	<.0001	0.71
FCR, kg/kg	4.21	4.05	0.117	0.32	<.0001	0.42

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by time interaction effect.

Table 5. Carcass quality from Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P-value*[2]
Item	C	BF	SEM	T
Age before slaughter, d	331.4	333.5	0.74	<0.05
Days in study, d	186.5	186.4	0.43	0.95
BW before slaughter, kg	460.7	460.7	3.44	0.99
Hot carcass weight, kg	243.8	244.2	2.07	0.91
Dressing percentage, %	52.9	53.0	0.26	0.81
Fatness, %				0.15
1				
2	30.1	41.7		
3	69.9	58.3		
Conformation, %				0.76
P	65.8	68.1		
O	32.9	29.2		
R	1.4	2.8		
U				

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect.

At the slaughterhouse BW, dressing percentage, carcass conformation and fatness classification were not affected by treatment (**Table 5**).

3.3. Animal behavior

All data for animal behavior, including both general activities and active behavior, are showed in **Table 6** for growing phase and **Table 7** for finishing phase.

General activities. No statistical differences were found in the percentage of animals per pen standing, lying, and drinking water during the visual observation period for the growing phase. Otherwise, the percentage of animals eating concentrate, eating straw and ruminating were greater ($P < 0.05$) for BF compared with C bulls.

During the finishing phase, again no differences during the visual observation period were found in the proportion of animals per pen standing, lying, and drinking water, but neither for animals eating straw. Once again, in this phase the proportion of animals eating concentrate and ruminating were greater ($P < .0001$) in BF bulls compared with C bulls.

Active behavior. In the growing phase, during the visual scan observation period, self-grooming and social behavior were not affected by treatment. On the other hand, oral non-nutritive behaviors were greater ($P < .0001$) performed by C compared with BF bulls, as well as all agonistic behaviors (fighting, butting, chasing and chasing-up) except displacement were also greater ($P < 0.05$) exhibited by C bulls than BF bulls. Regarding sexual behaviors, flehmen and attempt to mount were greater ($P = 0.01$) in C compared with BF bulls, although complete mounts were not affected by treatment.

During the finishing phase no differences between treatments were observed for social behavior, whilst bulls from the BF group performed ($P < 0.05$) more self-grooming than C bulls. Additionally, C bulls continued performing greater ($P < .0001$) oral non-nutritive behaviors than BF bulls during this phase. Again, differences among treatments in agonistic behaviors were found for this phase. Fighting, butting, and displacement were greater ($P < .0001$) exhibited by C bulls compared with BF bulls, and also C bulls tended

(P < 0.10) to show more chasing and chasing up behaviors than BF animals. In regard to sexual behaviors, C bulls performed greater (P < 0.001) flehmen behaviors, and tended (P < 0.10) to perform greater attempted and complete mounts compared with BF bulls.

Table 6. General activities (%) and social behavior (times/ 15 min) for growing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
General Activity, %						
Standing	75.02	74.64	0.031	0.84	<.0001	0.35
Lying	24.98	25.36	0.099	0.64	<.0001	0.31
Eating concentrate	8.80	11.99	0.058	<.0001	<.0001	0.45
Eating straw	9.96	14.30	0.062	<0.01	<0.05	0.95
Drinking	1.81	1.89	0.003	0.80	0.95	0.68
Ruminating	8.62	13.48	0.092	0.05	<0.05	0.38
Social behavior, /15 min						
Selfgrooming	21.81	24.14	1.075	0.13	<.0001	0.97
Social	2.83	2.61	0.667	0.64	<0.05	0.87
Oral non-nutritive	2.55	0.59	0.210	<.0001	<0.05	0.49
Fighting	8.02	3.83	0.917	<.0001	<0.001	0.86
Butting	3.19	0.86	0.110	<.0001	0.0001	0.12
Displacement	1.17	0.66	0.411	0.12	<0.001	<0.10
Chasing	0.95	0.06	0.267	<0.001	0.98	0.89
Chasing up	0.22	0.05	0.111	<0.05	0.44	0.62
Flehmen	1.89	1.30	0.146	0.01	<.0001	0.49
Attempt to mount	1.97	0.36	0.622	0.01	0.35	0.58
Complete mounts	3.16	1.88	0.935	0.14	<0.05	0.71

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.

[2] T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.

[3] SEM = standard error of the means of the log-transformed data (general activity) or root transformed data (social behavior).

Table 7. General activities (%) and social behavior (times/ 15 min) for finishing phase in Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

Item	Treatment[1]			*P*-values[2]		
	Control	BF	SEM[3]	T	Time	T x Time
General Activity, %						
Standing	62.66	60.90	0.075	0.68	<.0001	0.78
Lying	37.34	39.10	0.106	0.71	<0.001	0.84
Eating concentrate	5.56	9.81	0.035	<.0001	0.77	0.96
Eating straw	5.87	9.89	0.152	0.13	0.48	0.76
Drinking	1.45	1.68	0.006	0.46	0.41	0.85
Ruminating	9.72	15.97	0.126	<.0001	<0.001	0.17
Social behavior, /15 min						
Selfgrooming	11.75	15.25	1.127	<0.05	0.01	0.68
Social	4.04	5.63	0.848	0.32	<.0001	<0.10
Oral non-nutritive	3.29	0.92	0.282	<.0001	0.79	0.92
Fighting	10.46	4.53	0.803	<.0001	<.0001	0.49
Butting	5.50	0.92	0.664	<.0001	<0.05	0.15
Displacement	1.71	0.17	0.315	<.0001	0.96	0.84
Chasing	0.61	0.00	0.294	0.10	0.13	0.13
Chasing up	0.08	0.00	0.046	<0.10	0.11	0.11
Flehmen	4.42	2.08	0.464	<0.001	<0.10	0.85
Attempt to mount	2.24	0.58	0.756	<0.10	0.13	0.53
Complete mounts	2.92	1.25	0.130	<0.10	<0.01	0.50

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.

[2] T = treatment effect; Time = time effect (measurements every 14 d); T x Time = treatment by time interaction.

[3] SEM = standard error of the means of the log-transformed data (general activity) or root transformed data (social behavior).

3.4. Macroscopic rumen evaluation and liver abscesses

Results of the macroscopic evaluation at the slaughterhouse of rumen and liver are showed in **Table 8.** The color of rumen wall was lighter (P < 0.01) for BF bulls (72.22% classified as color < "3") compared with C bulls (46.58 classified as color < "3"). Papillae clumping in the rumen also tended (P < 0.10) to be greater in C bulls (16.44%) compared with BF bulls (6.94%). No differences among treatments were found

for the remaining macroscopic parameters analyzed at the slaughterhouse (liver abscesses and baldness areas).

Table 8. Macroscopical observations of the rumen and liver at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]		*P*-value[2]
Item	C	BF	
Color of the rumen[3]			< 0.01
1	9.59	22.22	
2	36.99	50.00	
3	53.42	26.39	
4	-	1.39	
Papillae clumping			< 0.10
Yes	16.44	6.94	
No	83.56	93.06	
Baldness region			0.28
Yes	39.73	48.61	
No	60.27	51.39	
Liver abscess[4]			0.40
None	79.45	79.17	
A	6.85	8.33	
A-	8.22	4.17	
A+	1.37	-	
Inflammation	2.74	8.33	

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect.
[3]Adapted from Gonzalez et al. (2001): Rumen color: 1= white; 5 = black.
[4]Adapted from Nocek et al. (1984).

3.5. Rumen pH and VFA concentration at slaughterhouse

The data of ruminal liquid parameters analyzed are presented in **Table 9.** Total VFA concentration and pH in the rumen were both affected by treatment. Thus, pH was greater ($P < 0.01$) in BF than in C bulls, and conversely total VFA concentration was greater ($P < 0.01$) for C bulls compared with BF bulls. The molar proportion of acetate was also affected by treatment, being greater ($P < 0.01$) in BF bulls compared with C bulls, whereas molar proportion of propionate was greater ($P < 0.01$) for C bulls than for

BF bulls. Furthermore, butyrate molar proportion was also greater (P = 0.05) in BF than in C bulls. The remaining of VFA analyzed (valerate, isobutyrate and isovalerate) were not affected by the treatment. Accordingly to acetate and propionate molar proportions, acetate:propionate ratio was greater (P < 0.05) for the BF bulls than for C bulls.

Table 9. Rumen VFA concentration at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Treatment[1]			*P-value*[2]
	C	BF	SEM	
Rumen				
pH	6.06	6.57	0.122	<0.01
Total VFA, m*M*	131.3	96.1	7.62	<0.01
Individual VFA, mol/100 mol				
Acetate	53.6	58.9	1.34	<0.01
Propionate	35.9	29.8	1.56	<0.01
Isobutyrate	0.8	0.9	0.09	0.72
n-butyrate	6.6	7.2	0.24	0.05
IsoValerate	1.7	1.7	0.24	0.90
Valerate	1.5	1.6	0.09	0.33
Acetate:propionate, mol/mol	1.6	2.1	0.12	< 0.05

[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids at 0.04%.
[2] T = treatment effect.

3.6. Expression of genes in the rumen and duodenum epithelium

The data of the relative gene expression at mRNA level in the rumen epithelium are presented in **Figure 1**. The supplementation with flavonoids affected the expression of all the *TAS2R* analyzed except *TAS2R38*. The relative gene expression of *TAS2R7*, *TAS2R16*, and *TAS2R39* was greater (P < 0.01) in the rumen of BF compared with C bulls. The relative gene expression of all receptors related with the neurotransmitter signaling studied (*ffar3* and *ffar2*, *adrac2c*, *ppyr1* and *cckbr*) was greater (P < 0.05) in BF bulls compared with C bulls. Furthermore, the relative gene expression of some receptors related with the inflammation like cytokine *IL-25*, *TLR4*, and *β-defensin*, was also greater (P < 0.05) for BF bulls than for C bulls.

Regarding the relative expression at mRNA level of genes studied in the duodenum epithelium, data are presented in **Figure 2**. Again, the supplementation with flavonoids affected the gene expression of all the *TAS2R* analyzed except *TAS2R38*.

Contrary to the results in rumen epithelium, in duodenum the relative gene expression of *TAS2R7, TAS2R16,* and *TAS2R39* was greater ($P < 0.001$) in C bulls compared with BF bulls. The relative gene expression of some receptors related with the neurotransmitter signaling also differ among treatments. The relative gene expression for *ffar2*, *ppyr1* and *cckbr* was greater ($P < 0.01$) for C than for BF bulls. Additionally, the relative gene expression of the receptors related with the inflammation such as *IL-25, TLR4*, and *β-defensin*, was again greater ($P < 0.05$) for C bulls than for BF bulls, contrary to rumen epithelium results.

4. Discussion

Citrus flavonoid supplementation has clearly reduced concentrate intake in bulls during the growing phase, although the remain performance parameters analyzed have not been affected throughout the study. Moreover, when bulls were supplemented with citrus flavonoids they spent more time eating concentrate than C bulls, but also devoted more time to eat straw and to perform ruminating activities during the scan visual procedure for the growing phase. In all our previous studies (Chapter III, IV and V), bulls supplemented with citrus flavonoids have devoted more time to eat concentrate during the growing phase but, with the exception of one study (Chapter IV), a decrease in concentrate intake has been mainly observed. Mechanisms whereby this eating regulation occur have been discussed previously (Chapter III, IV and V): i) increasing propionic acid production, and reducing acetate:propionate ratio in the ruminal liquid, affecting the eating pattern of bulls (Chapter III and V); ii) modifying the gene expression of bitter taste receptors (*TAS2R*) and anorexigenic peptides and hormones in rumen epithelium (Chapter IV, V).

In the present study, the fat content was increased during the finishing phase. As previously mentioned, supplementing fat is commonly used to increase energy density in finishing diets of fattening bulls (Krehbiel et al., 2006; Hess et al., 2008). However, high-fat diets modify eating pattern in cattle, and supplementing fat above 6% to 7% may reduce feed intake (Krehbiel et al., 2006; Devant et al., 2013). Furthermore, the type of fat and the degree of saturation of this fat can affect the extent of reduction in the feed intake of supplemented diets (Relling and Reynolds, 2007). Additionally, satiety hormones such as cholecystokinin (*cck*) and pancreatic polypeptide (*pp*) have been

associated with the reduction in feed intake related to high fat levels in cows (Choi and Palmquist, 1996; Relling and Reynolds, 2007). Previously, citrus flavonoid supplementation decreased the gene expression of *cckbr* and *ppyr1* in rumen epithelium when concentrate was fed in meal form (Chapter V). The release of these anorexigenic hormones, such as *cck*, neuropeptide Y (*npy*), and peptide YY (*pyy*), are triggered by the activation of *TAS2R* (Chen et al., 2006; Depoortere, 2013; Takai et al., 2016). Thus, it was expected that citrus flavonoid would decrease the gene expression of *TAS2R* when added to high-fat finishing concentrate in the present study, triggering the decrease in the gene expression of *cckbr* and *ppyr1* in rumen epithelium (as observed in Chapter V). However, completely contraty to expected, when citrus flavonoids were supplemented to high-fat (palm oil) concentrate the relative gene expression of almost all *TAS2R* analyzed in the rumen epithelium of bulls sharply increased.

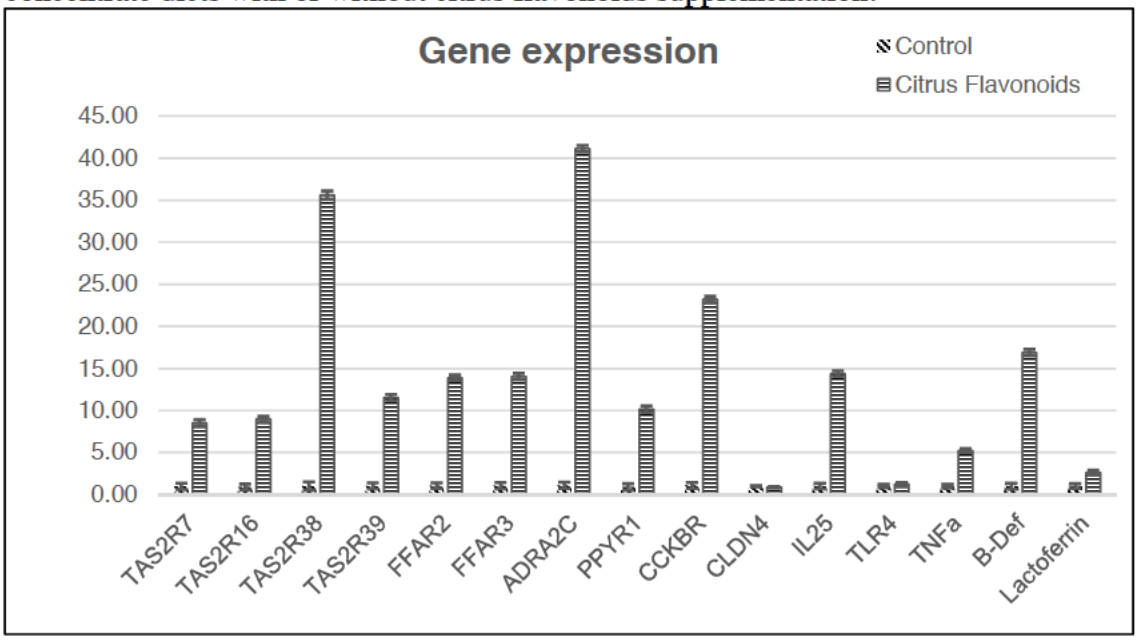
These results raises different questions, being the first one why high-fat (palm oil) concentrate resulted in the opposite effect in the expression of the genes analyzed in the rumen epithelium when citrus flavonoid were supplemented. One explanation could be that fats, and especially oils (in this study palm oil was used), exert negative effects on ruminal microflora growth, particularly affecting protozoa and fibrolytic bacteria (Enjalbert et al., 2017). Naringin, a bitter tasting glycosylated flavanone, is rapidly deglycosylated to naringenin by rumen microflora (Simpson et al., 1969; Cheng et al., 1971), and naringenin acts as an important bitter masking molecule (Jacob et al., 2014). Cheng et al. (1969), found that strains of ruminal *Butyrvibrio* spp hydrolyzed the glycosidic bond, metabolizing naringin to naringenin in rumen. Interestingly, recent research has showed that *Butyrbibrio* spp growth is clearly inhibited by the presence of oils and fats at low concentrations (Enjalbert et al., 2017). Consequently, it might be hypothesized that high-fat diets might interfere deglycosylation of naringin to naringenin by rumen bacteria as *Butyrvibrio* spp. Thus, that would help to explain the higher gene expression of *TAS2R* in ruminal epithelium of bulls supplemented with citrus flavonoids.

Probably due to this increase in the gene expression of *TAS2R*, the production, expression, and turnover of the anorexigenic neurotransmitters' receptors studied were also greater expressed in bulls supplemented with citrus flavonoids. The differences in the expression of different genes would support a decrease feed intake. First of all, as previously mentioned, activation of *TAS2R* triggers the release of anorexigenic molecules

as *cck* and *pyy* (Damak, 2016; La Sala et al., 2013; Takay et al., 2016), and also fatty acids are been related with an increase in *cck* and *pp* in ruminants (Choi and Palmquist, 1996; Relling and Reynolds, 2007). Secondly, in this study, the gene expression of *adrac2r* in rumen epithelium of BF bulls was greatly increased, and this receptor induces a reduction in forestomach contractions (Meylan et al., 2004) that could lead to a reduction in concentrate intake reducing the passage of ruminal content to the abomasum and intestines. Finally, specific nutrient-sensing receptors for fats, the *ffar*, have been described throughout the digestive tract of different animal species (Mielenz, 2016): *ffar1* (*GPR40*), activated by long and medium chain fatty acids; *ffar2* (*GPR43*), activated by short chain fatty acids; *ffar3* (*GPR41*), also activated by short chain fatty acids; and, finally, *ffar4* (*GPR120*), activated specifically by long chain fatty acids. All these *ffar* are found in enteroendocrine cells, and are responsible for the secretion of different anorexigenic molecules such as *glucagon-like peptide-1* (*GLP-1*), *ppy* and *cck* (Damak, 2016; Mielenz, 2016). Our results showed an increase in *ffar2* and *ffar3* gene expression in bulls supplemented with citrus flavonoids, whilst VFA analyzed at slaughterhouse clearly exhibited a reduction in total VFA concentration in BF bulls, and a reduction in molar proportions of propionate as well. Recent research has demonstrated interactions between bitter and lipid sensing (La Sala et al., 2013), so it could be hypothesized that citrus flavonoids supplementation to bulls fed high-fat diets might increase the gene expression of *ffar 2* and *ffar3* due to an interaction among bitter and fat nutrient-sensing pathways.

Actually, bitter taste and fat seems to be both determinant pathways playing a key role in satiety mechanisms in the animals, but for different reasons. In fact, bitter taste could be considered as one of the main sensory defense mechanisms in animals to avoid the ingestion of toxic substances, so it make sense that the activation of *TAS2R* throughout the digestive tract triggers the activation of anorexigenic molecules and pathways sending signals to the brain to stop eating. On the other hand, fats are the nutrients with the highest energy value, triggering also important cues that activates satiety. Indeed, both bitter taste and fats reduce meal size in bulls (Krehbiel et al., 2006; Devant et al., 2013; Chapter III). Consequently, supplementing citrus flavonoids to bulls fed high-fat diets could have exacerbated the release of anorexigenic hormones and peptides, explaining the results observed in the gene expression in the rumen epithelium.

Figure 1. Gene expression in rumen epithelium of Holstein bulls fed high-concentrate diets with or without citrus flavonoids supplementation.



TAS2R7: *Bitter taste receptor 7;* TAS2R16: *Bitter taste receptor 16;* TAS2R38: *Bitter taste receptor 38;* TAS2R39: *Bitter taste receptor 39;* FFAR2: *Free fatty acid receptor 3 (gpr41);* FFAR3: *Free fatty acid receptor 2 (gpr43);* ADRA2C: *Alpha 2-adrenergic receptors subtype C;* PPYR1: *Pancreatic polypeptide receptor 1;* CCKBR: *Cholecystokinin receptor 4;* CLDN4*: Claudin4;* IL-25: I*nterleukin-25;* TLR4: *Pattern recognition receptors, like Toll-like receptor 4;* TNFa: *Tumor necrosis factor alpha;* B-Def: *Beta-defensin*. The values presented herein correspond to back-transformed means; however, SEM correspond to the ANOVA analyses using log-transformed data.

The second main question would be why if relative expression in the rumen epithelium of genes related with eating pattern was so different among treatments (**Figure 1**) during the finishing phase no differences in feed intake (amount) among treatments were observed. May be the answer could be related with the gene expression in duodenum that was reduced for almost all nutrient-sensing receptors studied in bulls supplemented with citrus flavonoids compared with C bulls, including *TAS2R* and anorexigenic receptors (such as *ffar2*, *cckbr*, *adra2c* and *ppyr1*) (**Figure 2**). Surprisingly, these results would be in agreement with the results observed in rumen epithelium of our previous study (Chapter V), when the concentrate was not supplemented with large quantity of fat (palm oil). But these results would imply that naringin should have been deglycosylated to naringenin just before arriving or at arriving to the duodenum, acting in this part of the intestine as a bitter masking molecule and blocking the *TAS2R*, along with the anorexigenic molecules released by these receptors. Certainly, these changes in gene expression observed in duodenum epithelium might be also affecting feeding behavior of

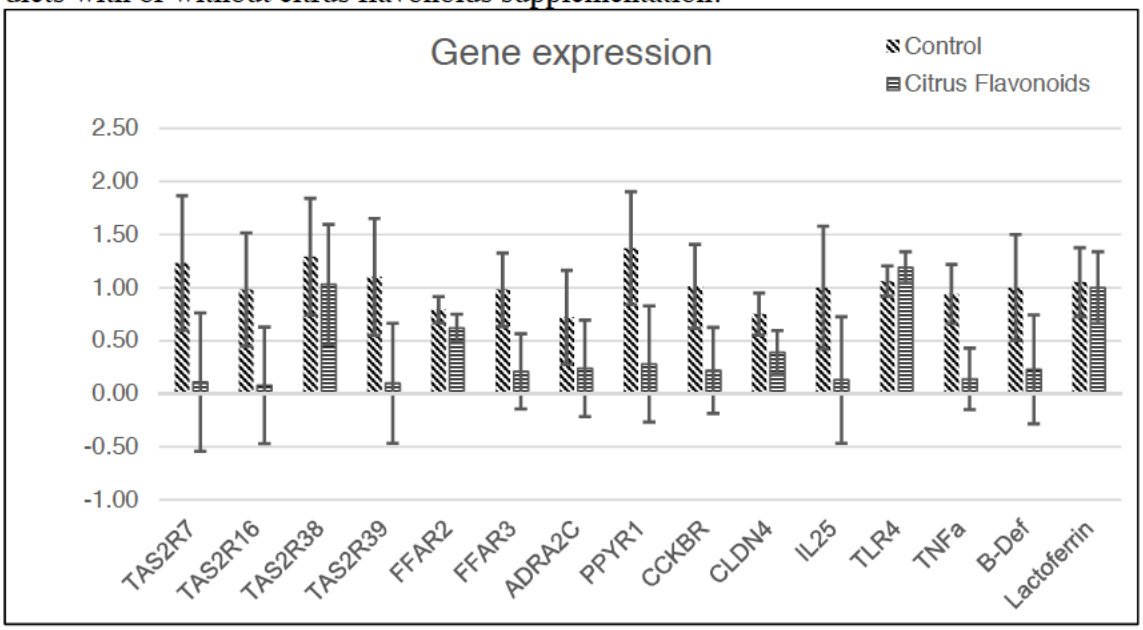
these bulls, along with differences observed in rumen epithelium. In fact, during the finishing phase, when high-fat concentrate was fed, again BF bulls devoted more time to eat concentrate and also to perform greater ruminating activities during the visual scan procedure. Actually, all these mechanisms have been described as important part of the gut-brain axis network regulating eating pattern in animals, but much more research is needed to properly describe the importance of the digestive tract site (rumen, intestine), if these effects are additive across the different parts of the digestive tract, and how they might be modulating feeding behavior, hunger and satiety in bulls.

As previously mentioned, citrus flavonoid supplementation clearly decreased total VFA in ruminal liquid at slaughterhouse, and increased ruminal pH. Also acetate molar proportion was increased reducing propionate, so acetate:propionate ratio was higher for BF bulls. Conversely, the gene expression of *ffar2* and *ffar3* was higher for BF bulls, as formerly discussed. Previous research carried out with citrus flavonoids also showed an improvement in ruminal pH due to a ruminal microflora modulation, but the concentration of ruminal propionate was higher when citrus flavonoids were supplemented (Balcells et al., 2012). These differences could be explained because of: i) the extract of citrus flavonoids and dose supplemented were different; ii) sampling the ruminal liquid in-farm or at the slaughterhouse can affect ruminal VFA concentration and profile (Lam et al., 2018); and iii) also the high-fat concentrate fed in this study could have affected VFA production in the rumen. This greater ruminal pH along with lower VFA concentration could explain that macroscopic rumen wall parameters studied at the slaughterhouse were greater in BF bulls. Rumen wall color was lighter and baldness area were reduced in bulls supplemented with citrus flavonoids, and that might be indicative of better ruminal health.

In agreement with our previous research (Chapter III, IV and V), citrus flavonoids supplementation modulated animal behavior, reducing oral non-nutritive behaviors, aggressive and sexual interactions. In beef cattle, oral non-nutritive behavior and aggressive interactions are indicators of poor welfare and stress (Gonyou et al., 1994; Bergeron et al., 2006). Thus, oral non-nutritive behavior has been related with digestive dysfunctions, as low pH, ruminal lesions or even with the impossibility of performing natural eating behaviors as rumination in beef cattle (Bergeron et al., 2006). Our results have showed an improvement in macroscopical ruminal wall parameters as lighter color

and lower presence of baldness areas, along with an increase in ruminal pH when bulls were supplemented with citrus flavonoids. Additionally, BF bulls also performed greater ruminating activity during the visual scan procedure. Therefore, in this study citrus flavonoids might have reduced oral non-nutritive behaviors by improving ruminal health, increasing ruminal pH and rumination activities.

Figure 2. Gene expression in duodenum of Holstein bulls fed high-concentrate diets with or without citrus flavonoids supplementation.



TAS2R7: *Bitter taste receptor 7;* TAS2R16: *Bitter taste receptor 16;* TAS2R38: *Bitter taste receptor 38;* TAS2R39: *Bitter taste receptor 39;* FFAR2: *Free fatty acid receptor 3 (gpr41);* FFAR3: *Free fatty acid receptor 2 (gpr43);* ADRA2C: *Alpha 2-adrenergic receptors subtype C;* PPYR1: *Pancreatic polypeptide receptor 1;* CCKBR: *Cholecystokinin receptor 4;* CLDN4*: Claudin4;* IL-25: I*nterleukin-25;* TLR4: *Pattern recognition receptors, like Toll-like receptor 4;* TNFa: *Tumor necrosis factor alpha;* B-Def: *Beta-defensin*. The values presented herein correspond to back-transformed means; however, SEM correspond to the ANOVA analyses using log-transformed data.

In the present study citrus flavonoids supplementation reduced all agonistic and sexual interactions. Inflammation, neuropeptides but also nutrient sensing mechanisms such as *TAS2R* and *ffar* have been proposed for this animal behavior modulation by citrus flavonoids supplementation through the gut-brain axis cross-talk mechanisms (Chapter IV and V). In our previous study carried out with concentrate in meal form (Chapter V), citrus flavonoids supplementation clearly reduced the gene expression of some proinflammatory molecules in the rumen epithelium (*IL-25, TLR4*, and *β-defensin*), whilst some of them increased when concentrate was fed in pellet form (Chapter IV). Surprisingly, the gene expression of these proinflammatory molecules (*IL-25, TLR4*, and

β-defensin) in the rumen epithelium were clearly greater expressed in BF bulls in the present study. Fatty acid excess has been related with inflammation through the activation of *TLR-4*, which is also involved in innate immune response, and triggers the secretion of proinflammatory cytokines (Yunhe et al., 2013; Yamashita et al., 2018). Additionally, the gene expression in the rumen of the *TAS2R* analyzed was also higher in BF bulls, and these receptors also play important roles in the immune response (Ahmed et al., 2016). Consequently, this increase in proinflammatory molecules in the rumen epithelium when citrus flavonoids were supplemented in bulls fed high-fat diets would be result of *TAS2R* gene expression increase, but also high-fat level of the concentrate would be triggering this proinflammatory response. On the other hand, the gene expression of these proinflammatory molecules in the duodenum epithelium of BF bulls was reduced, as observed in the rumen epithelium of our previous study (Chapter V). These results were also in agreement with the reduction of the nutrient sensing receptors studied in the duodenum epithelium of BF bulls, such as *TAS2R*, *ffar2*, *adra2c*, *ppyr1*, and *cckbr*. Thus, as previously discussed, maybe naringin is deglycosylated to naringenin just before or at arriving to the duodenum, exerting its effects as bitter masking molecule and as a potent antioxidant. Consequently, with the present results of gene expression in rumen and duodenum epithelium, and also considering our previous results (Chapter IV and V), it is difficult to conclude the gut-brain axis mechanisms whereby citrus flavonoids might be modulating animal behavior.

As in our previous studies (Chapter III, IV and V), eating pattern modulation observed in bulls when supplemented with citrus flavonoids could be leading to a reduction of aggressive and abnormal behaviors by increasing time devoted to feeding events, as eating concentrate, straw and rumination.

In summary, animal and eating behavior results were closer to previous studies (Chapter III, IV and V). However, when bulls were fed high-fat (palm oil) diets supplemented with citrus flavonoid, the gene expression was the opposite in the rumen epithelium than observed in mash diets without high-fat levels (Chapter V). Additionally, the gene expression of duodenum epithelium was similar to observed in ruminal epithelium when mash diets without high-fat levels were fed to Holstein bulls (Chapter V).

5. Conclusions

In conclusion, during the growing phase citrus flavonoids reduced concentrate intake in bulls fed high-concentrate in meal form. Moreover, citrus flavonoids reduced oral non-nutritive behaviors, agonistic interactions and sexual behaviors in bulls, potentially improving animal welfare. The macroscopical study of the rumen wall suggested that rumen health was better when citrus flavonoids were added to the concentrate, also improving ruminal pH. Moreover, flavonoid supplementation differently modified the expression of genes in the rumen and duodenum epithelium that could be related with eating pattern and animal behavior regulation.

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CHAPTER VII

General Discussion

The discussion of the present work has been structured in four parts. The first part is focused on the practical implications of supplementing citrus flavonoids in fattening bulls under commercial conditions to improve profitability, based on the results of the four studies performed (Chapter III, IV, V, and VI). Secondly, some hypothesis of possible nutrient-sensing mechanisms involved in the modulation of the eating pattern in bulls supplemented with citrus flavonoids are discussed. In the third part, the elaboration of different hypothesis of how citrus flavonoids could be modulating animal behavior are discussed, analyzing jointly the results of all four studies. Finally, brief comments and personal thoughts about future research on gut-brain axis, nutrient sensing and chemosensory transduction in cattle are suggested.

1. PRACTICAL IMPLICATIONS OF THE RESULTS

1.1. Practical overview of the results

From the beginning, one of the most important objectives of this thesis was to evaluate the practical application of citrus flavonoids extracted from *Citrus aurantium* in beef cattle raised in typical commercial conditions in our intensive production system. That's the main reason why studies were carried out with Holstein bulls in adapted commercial farms that allowed the record of animal growth and concentrate intake. In the Mediterranean countries, due to weather conditions, forage availability is scarce and expensive as well, so our intensive beef production systems are mainly based on the use of high-concentrate feedstuffs rich in cereals, using mostly straw from different cereals as a fiber source. Thus, feeding concentrate plays a crucial role in the productive cost, and reducing concentrate intake without impairing productivity is an interesting challenge to improve efficiency and also economic profits.

To facilitate this discussion, **Table 1** shows a summary of the performance results to analyze the practical implications. As the intention is to perform an analysis from a practical point of view, this table contains numerical results, and not all of them have been statistically different compared with the control. It is always quite difficult to obtain statistical differences in performance parameters. Basically, Holstein bulls are considered a by-product of dairy cattle production, and animals are collected from different origins, from different farms and even from different countries. So the high degree of genetic

variability existing within bulls fattened under the same conditions, which affects their genetic traits for performance and productivity, makes quite difficult to obtain statistical differences when working with these animals. In monogastric species or dairy cattle the animal variability is not so great. Numerical differences can give us some interesting data from the economical point of view although they should be interpreted with caution.

In this **Table 1,** results have been divided in three main blocks of data: performance, animal behavior and carcass results. Furthermore, productive phases have been considered as well: growing, finishing, and all fattening period. Green color indicates considered as positive results of the supplementation with citrus flavonoids compared with non-supplemented bulls within the same trial, whereas red color indicates completely the opposite (considered as negative effects in bulls when citrus flavonoids are supplemented). Orange color indicates that it is not clearly interpreted as positive or negative effect.

Regarding performance parameters, in general the most consistent effect observed with citrus flavonoids supplementation is the reduction in concentrate intake in three of the four trials performed (excepting for the trial performed with pellet in a multi-space feeder) mainly during the growing period. In fact, the only negative effect of supplementing citrus flavonoids in bulls was observed when the single-space feeder was used to study the eating pattern (Chapter III), reducing the final BW of the animals and, as discussed in Chapter IV, this could be due to the limitation of feeder occupancy. In the studies performed using the concentrate in meal form (Chapter V and VI), animals reduced their concentrate intake without impairing their performance, so these bulls were more efficient when supplemented with citrus flavonoids although FCR was not statiscally different. Additionally, citrus flavonoids supplementation did not affect HCW at the slaughterhouse in any study. Although fat classification of the carcass in the study carried out using concentrate in pellet form was statistically affected, it is quite difficult to assess if this result is positive or negative looking at the numbers.

Table 1. Summary of a practical overview of the results of the different studies.

Phase	Parameter	Single-feeder Chapter III	Pellets Chapter IV	Meal Chapter V	Meal High-fat Chapter VI
Performance[1]					
Growing phase	BW	=	=	=	=
	Intake	↓ ***2.3%***	=	↓ 3.4%	↓ **5.4%**
	ADG	=	=	=	=
	FCR	=	=	↓ 3.5%	↓ 5.3%
Finishing phase	BW	↓**1.8%**	=	=	=
	Intake	↓ 2.6%	=	=	=
	ADG	=	=	=	=
	FCR	=	=	↓ 5.9%	=
All fattening period	BW	↓**1.8%**	=	=	=
	Intake	↓ **2.5**	=	↓ 2.4%	↓ 4.1%
	ADG	=	=	=	=
	FCR	=	=	↓ ***4.4%***	↓ 3.8%
Behavior[2]					
General behavior, percentage					
Growing phase	Eating concentrate	↑	↑	↑	↑
	Eating straw	↑	=	=	↑
	Ruminating	=	↑	=	↑
Finishing phase	Eating concentrate	↑	↑	↑	↑
	Eating straw	↑	=	=	=
	Ruminating	=	↑	↑	↑
All fattening period	Eating concentrate	↑	↑	↑	↑
	Eating straw	↑	=	=	↑
	Ruminating	=	↑	↑	↑
Active behavior, number of events in 15 min					
Growing phase	Oral non-nutritive	↓	=	↓	↓
	Aggressive	↓	↓	↓	↓
	Sexual	=	=	↓	↓
Finishing phase	Oral non-nutritive	↓	↓	=	↓
	Aggressive	↓	↓	↓	↓
	Sexual	↓	↓	↓	↓
All fattening period	Oral non-nutritive	↓	↓	↓	↓
	Aggressive	↓	↓	↓	↓
	Sexual	↓	↓	↓	↓
Carcass					
	HCW	=	=	=	=
	Fat classification	=	??	=	=

[1] **highlighted in bold**: statistical differences between treatments within the same study.***Highlighted in bold and italics***: statistical tendency, between treatments within the same study. Percentages considered when numerical differences were > 2% (unless for statistical differences).

[2] Only statistical differences are considered.

Probably the most consistent and unfluctuating results among studies is observed in the animal behavior, in activities related to the eating pattern and also in the active behavior studied. In regard to eating pattern, in most studies performed and during the different productive phases, bulls supplemented with flavonoids devoted more time to eat concentrate. On the other hand, eating straw and ruminating activities varied between the different studies, basically between the single-space feeder study (Chapter III) and the rest (Chapter IV, V and VI). When the single-space feeder was used, bulls supplemented with flavonoids devoted more time to eat straw than non-supplemented bulls. In the remaining studies (performed with a multi-space feeder) bulls supplemented with flavonoids dedicated more time to rumination activities compared with control bulls, but time devoted to eat straw was not different between groups. In the case of the multi-space feeders bulls supplemented with flavonoids devoted more time to eat concentrate, although their concentrate intake was lesser compared with non-supplemented bulls and the percentage of animals eating straw did not differed between treatments. The single-feeder may have been limiting the access to concentrate feed and animals would have redirected their activity to eat straw as discussed in Chapter IV.

Citrus flavonoids supplementation in bulls reduced oral non-nutritive behavior, aggressive and sexual interactions. This response was independent of the study, the feeder space or the concentrate presentation (pellet or meal) or composition, indicating a consistent improvement in well-being of bulls supplemented with citrus flavonoids.

On the other hand, when talking about feed additives, it is always required to evaluate the economic return of their addition, because it supposes an extra cost. Thus, a brief and simple evaluation of supplementing citrus flavonoids in the concentrate of bulls is showed in **Table 2**. Animal behavior has not been taken into account in this evaluation. As feed concentrate and citrus flavonoids prices are variable, this table shows kg of concentrate save per bull when citrus flavonoids were supplemented. Thus, depending on economical circumstances citrus flavonoids supplementation would be more or less economically interesting.

These numbers are been calculated per bull, and for the four studies performed. Based in these results, the supplementation with citrus flavonoids would be economically viable when feeding the concentrate in meal form, depending on market prices for concentrate and, obviously, for the citrus flavonoids. Conversely, only based on

performance parameters and economic profits, adding citrus flavonoids to the concentrate in pellet form would suppose an extra cost, using both the single-space feeder or multi-space feeder.

Table 2. Study of the economic viability of using citrus flavonoids in commercial farms.

	Parameter	Single feeder Chapter III	Pellets Chapter IV	Meal Chapter V	Meal High-fat Chapter VI
Growing phase	Days	112	112	112	112
	Intake/ day (g)	**↓150**	**↓ 40**	**↓223**	**↓330**
	Concentrate, kg	**↓16.8**	**↓4.5**	**↓25**	**↓37**
Finishing phase	Days	56	56	56	56
	Intake/ day (g)	**↓200**	**↓ 130**	**↓ 50**	**↓ 140**
	Concentrate, kg	**↓11.2**	**↓7.3**	**↓2.8**	**↓7.8**
Total study	Concentrate, kg	**↓28**	**↓11.8**	**↓27.8**	**↓44.8**

1.2. Practical recommendations for the use of citrus flavonoids in fattening bulls fed high-concentrate diets

As previously mentioned, at the beginning of this work one important objective was to develop a commercial application for citrus flavonoids extracted from *Citrus aurantium* in beef cattle produced under intensive production conditions, so basically fed high-concentrate diets rich in cereals and straw as fiber source. Thus, based on previous research carried out with citrus flavonoids (Balcells et al., 2012; Seradj et al., 2014), the first study performed (Chapter III) had the aim of studying the possible effects of supplementing citrus flavonoids over eating pattern, productive parameters and animal behavior in bulls. To accomplish this objective, the study was conducted in a commercial farm adapted with a single-space feeder per pen designed to register individually the eating pattern of every bull. After that, the second study (Chapter IV) was carried out in a commercial farm using multi-space feeders, and concentrate was fed in pellet form like in the first study. So here the aim was to evaluate if the space feeder would have been limiting the performance in bulls supplemented with citrus flavonoids when a single-space feeder was used. Following this, the third study (Chapter V) was designed exactly like the previous one, the same farm and feeders, but concentrate was fed in meal form. Basically, the objective was to analyze if the presentation form of the concentrate was

affecting the eating pattern of bulls supplemented with citrus flavonoids. Finally, the last study was again carried out in the same commercial farm, also the same multiple-feeders were used, but in this case a high-fat finishing concentrate in meal form was fed to the bulls. So, a possible interaction between citrus flavonoids and fat content of the feed formula was investigated.

Obviously, the study of all the possible combinations and variations that could be found in commercial conditions were impossible to replicate, but the space of the feeder, the concentrate presentation and the fat content of the formula would offer interesting and useful information for practical application of citrus flavonoids in commercial farms.

1.2.1. Feeder space

In Chapter III, the use of the single-space feeder allowed us to find an important difference in the eating pattern when bulls were supplemented with citrus flavonoids, reducing the percentage of large meal sizes (> 750 g) during the finishing phase. Although Verdú et al. (2015) found that using this single-space feeder animals reduced concentrate intake without impairing performance, when citrus flavonoids were supplemented bulls decreased their concentrate intake, but also the final BW was reduced. Normally, an increase in the number of visits to the feeder have been found in bulls when meal size is reduced, to compensate the intake (Devant and Bach, 2017), and probably due to the use of the single space feeder bulls were not able to increase enough the number of meals. In Chapter IV, concentrate in pellet form was fed using a three space feeder, and bulls supplemented with citrus flavonoids increased the time devoted to eat concentrate, and they would have compensated the concentrate intake and final BW, and no differences were found with non-supplemented bulls.

Through all the studies performed, the most consolidate effect over the eating pattern observed in bulls supplemented with citrus flavonoids was that these animals occupied during more time the concentrate feeder, independently if it was the single or multiple-space feeder (unless in the finishing phase of Chapter IV). Furthermore, performance parameters studied were not impaired by citrus flavonoids supplementation when multiple-space feeder was used. In fact, the visual scan procedure showed that bulls supplemented with citrus flavonoids when a single-space feeder was used clearly devoted

more time to eat straw, and this effect was not observed in the rest of our studies performed with multiple space feeders (Chapter IV, V and VI), except for the growing phase of the Chapter VI. So, probably these bulls redirected their eating behavior to eat straw when they were not able to access to the concentrate feeder.

Therefore, when citrus flavonoids are added to the concentrate the feeder space per bull would be crucial, as these animals would devote more time to eat and reduce the large meal sizes, so the number of visits to the feeder will increase. Although our studies do not allow to make a recommendation of the ideal ratio of bulls per feeder space, when the three-space feeder was used (6 bulls per feeder space) no differences in final BW and HCW were found compared with control group, so this ratio could be considered as sufficient not to impair performance.

1.2.2. Concentrate presentation

Based on the results of our studies, when bulls were fed with concentrate in meal form (Chapter V and VI) supplementation with citrus flavonoids showed a positive effect in bulls, reducing concentrate intake but achieving the same final BW and HCW than non-supplemented animals. Actually, in Chapter V bulls supplemented with citrus flavonoids tended to be more efficient (lesser FCR) throughout the study. Conversely, in Chapter VI this difference in FCR was only numerical at the end of the study, probably due to the high-fat concentrate fed during the finishing phase, because during the growing phase results in FCR of both studies were very similar (when the same formula and presentation of the concentrate were used). Otherwise, when concentrate was fed in pellet form (Chapter III and IV) no differences of supplementing citrus flavonoids were observed in performance parameters. How the concentrate presentation would be affecting citrus flavonoids effects will be discussed later with the eating pattern modulation by citrus flavonoids.

Consequently, from the point of view of performance and based in our studies, supplementing citrus flavonoids when concentrate is fed in meal form would have positive effects on concentrate efficiency, reducing concentrate intake without impairing performance parameters (final BW and HCW).

1.2.3. Formula composition

Increasing the percentage of fat in the concentrate fed during the finishing phase of fattening bulls in intensive production systems is commonly used to modulate feed intake but also to increase energy density of the feed formula ((Heinrichs et al., 1982; Hess et al., 2008; Devant et al., 2013; Martí et al., 2014) or to add feed additives that are heat sensitive like some vitamins or yeast. As supplementing fat above 6% to 7% may reduce feed intake reducing the meal size in cattle (Krehbiel et al., 2006; Devant et al., 2013), as observed in Chapter III when bulls were supplemented with citrus flavonoids, an interaction between fat level and citrus supplementation was expected.

In Chapter VI, this possible interaction was studied, and no effect was observed in high-fat concentrate intake due to the supplementation with citrus flavonoids. Furthermore, performance parameters analyzed were also not affected by citrus flavonoids in this study. Consequently, the supplementation of high-fat concentrates with citrus flavonoids would not reduce concentrate intake in bulls beyond effects of fat level, and would not improve performance either. Thus, supplementing both fat and citrus flavonoids are not incompatible, but deciding using fat, citrus flavonoids or both would depend on the objective at farm. It would make sense to use both when trying to improve animal welfare, but not when looking for reducing costs or improving concentrate efficiency.

1.3. Citrus flavonoids supplementation to improve animal welfare

Supplementing citrus flavonoids to fattening bulls fed high-concentrate diets consistently modulated animal behavior throughout the different studies carried out. In spite of the limitations of the visual scan procedure, which was performed during a limited period in the morning, abnormal oral behaviors, aggressive and sexual interactions were reduced in a different extend in the different studies.

Throughout the different studies, citrus flavonoids supplementation reduced aggressive interactions during the growing phase, being this effect more evident during the finishing phase. Also for sexual interactions, reduction observed when citrus flavonoids were supplemented become more evident during this finishing phase. In beef cattle, and especially in Holstein bulls, aggressive and sexual behaviors are an important

welfare problem especially in this finishing phase, when bulls achieve their sexual maturity (age around 7 months old). These interactions suppose a risk of physical damage (limps due to the mounts and different injuries because of beating behaviors), but also may impair performance of the bulls, basically due to the energy waste and stress. Castration has been proposed as a solution for these behaviors, but it supposes animal welfare implications and a reduction in bulls' efficiency. Consequently, citrus flavonoids would be a very useful alternative to reduce these behaviors without impairing animal performance and well-being. Furthermore, independently on the feeder system, and concentrate presentation or composition, citrus flavonoids have reduced aggressive and sexual interactions, even more improving bulls efficiency in some cases (Chapter V and VI). In these studies citrus flavonoids have been continuously supplemented during growing and finishing phase. Consequently, supplementing citrus flavonoids exclusively during the finishing phase to reduce sexual and aggressive interactions in bulls should be studied considering the positive effects observed.

On the other hand, oral non-nutritive behaviors have been reduced in bulls supplemented with citrus flavonoids as well. Licking or biting fixtures with non-nutritive finality has been described as an stereotypic behavior in cattle, and digestive dysfunctions, ruminal acidosis and frustration have been proposed as possible causes for this type of behavior (Bergeron et al., 2006). So, reducing the performance of these oral behaviors might be related with an improvement in animal welfare and digestive health. Therefore, supplementing citrus flavonoids in fattening bulls would also improve bulls' welfare by reducing this kind of stereotypic behavior, being probably a result of animal digestive health improvement.

Based in our results, supplementing citrus flavonoids throughout all the fattening period in Holstein bulls would improve animal welfare and, in some cases, animal performance as well. Consequently, citrus flavonoids would have a positive economic impact, also helping to face a society important demand, as animal welfare. More studies would be required to know if the same improvement would be obtained supplementing citrus flavonoids for a shorter period of time.

1.4. Summary of practical recommendations of citrus flavonoids supplementation:

2. To improve performance:
    - ✓ Adequate feeder space. Could be limiting the access to concentrate of the bulls, especially during the growing phase, when animals eating behavior is more gregarious.
    - ✓ Concentrate presentation in meal form.
    - ✓ During the growing phase, concentrate intake reduction without impairing bulls performance is greater than for the finishing phase.
    - ✓ No negative interaction is expected increasing fat level when supplementing citrus flavonoids.

3. To improve animal behavior:
    - ✓ Supplementing citrus flavonoids during the fattening period, independently of the feeder design, concentrate presentation, and concentrate fat level, will reduce non-oral behaviors, and aggressive and sexual interactions in Holstein bulls.

1. EATING PATTERN MODUALTION IN BULLS BY CITRUS FLAVONOIDS

Throughout the four studies performed in this thesis, an eating pattern modulation has been observed when citrus flavonoids were supplemented to the Holstein bulls, independently of the feeder design, the concentrate presentation or fat level inclusion in the concentrate formula. In some studies (Chapter III, V and VI), a modulation in the time devoted to eat, concentrate or straw, have been accompanied with a numerical but consistent reduction in concentrate intake, especially during the growing phase. On the other hand, in Chapter IV when concentrate was fed in pellet form in a multi-space feeder, again bulls supplemented with flavonoids spent more time eating concentrate, although no effect was observed in the concentrate intake of these animals.

Different hypothesis have been proposed and studied along the different chapters of this thesis, and these hypothesis have also influenced the different studies performed in order to deeper understand how these citrus flavonoids could be affecting bulls' eating behavior.

In our first study, carried out with the single-space feeder (Chapter III), among other effects, a reduction in large meal sizes during the finishing phase were observed. Furthermore, although no differences in the meal size, meal duration, eating rate or visits to the feeder were observed during the growing phase in this study, the time between meals was numerically increased in 4 minutes in bulls supplemented with citrus flavonoids, explaining the numerical reduction in concentrate intake observed. Consequently, three hypothesis were proposed fot this modulation of the eating behavior observed (less large meal size, more time devoted to eat concentrate):

- ✓ Bitter taste of citrus flavonoids.
- ✓ Higher propionic acid production in rumen when citrus flavonoids were supplemented.
- ✓ Meal size reduction limited concentrate intake due to the single-space feeder.

As ruminants possess a high threshold for bitter taste (Glendinning, 1994), the possibility that taste was limiting the intake at mouth level was quickly rejected, but the possible action of bitter molecules as flavonoids (Ribeiro et al., 2008) in the digestive tract was considered, due to the recent research in other mammals describing the presence of bitter taste receptors (TAS2R) throughout the digestive tract (Behrens and Meyerhof, 2011). The second hypothesis, higher propionic acid production in the rumen when citrus flavonoids were supplemented, was also considered. Balcells et al. (2012) found in cannulated heifers fed high-concentrate diets in meal form that VFA production was affected by citrus flavonoids supplementation, increasing propionate percentage and also reducing actetate:propionate ratio in the ruminal liquid. As propionate produced in the rumen has been described as a feed intake regulator in ruminants (Bradford and Allen, 2007), this was an important mechanism of action to be considered. Finally, the result observed was a reduction in concentrate intake, and bulls supplemented clearly devoted more time to eat straw in this study, so it was also quite important to study the possibility of the concentrate intake reduction due to the limitation of access to the concentrate because of the single-space feeder used to study the eating behavior.

In Chapter IV a multiple-space feeder were used, with the same feed formula also in pellet form than in Chapter III. The main objective was to study if bulls would be able to compensate meal size reduction increasing feeder visits. Also ruminal liquid was taken

at the slaughterhouse for the study of VFA, and samples of ruminal epithelium to study the expression of some genes related to nutrient sensing and bitter taste. Simultaneously, the study of the Chapter V was performed. With the same multiple-space feeder, but the concentrate was fed in meal form, to study the possible impact of concentrate presentation in citrus flavonoids effects. Ruminal liquid for VFA and ruminal epithelium for gene expression study were taken at the slaughterhouse as well. Finally, in the Chapter VI, and based on some of the results of these previous chapters, the possible interaction with fat level in concentrate was studied. In addition to ruminal liquid sample for VFA and ruminal epithelium for gene expression, samples of duodenum epithelium were also taken for the gene expression study.

Contrary to expected, in our studies ruminal propionate percentage were not higher in bulls supplemented with citrus flavonoids in the samples taken at the slaughterhouse (**Table 3**). In fact, when concentrate was fed in meal form (Chapter V and VI), propionate were statistically higher in control bulls, also total VFA content was higher in non-supplemented bulls, and acetate:propionate ratio was lower in these animals. Indeed, our results when concentrate was fed in meal form were completely the opposite of the obtained by Balcells et al. (2012) when also high-concentrate diets in meal form were fed to heifers. In any case, our results are not enough to reject this hypothesis of the effect of ruminal propionate as a key factor modulating concentrate intake when citrus flavonoids are supplemented, because differences could be found in VFA content and percentage depending on farm-sampling or taking the ruminal liquid sample at the slaughterhouse (Lam et al., 2018). Actually, in the study carried out by Balcells et al. (2012) samples of ruminal liquid were taken after weighting the cannulated animals at 8 am, so probably these heifers had just eaten concentrate. In our studies, animals were picked up at the farm around 12 pm, and slaughtered around two hours later, so it would be coherent that the fermentation parameters would be quite different between the studies due to methodology. Furthermore, macroscopic study of rumen wall when citrus flavonoids were supplemented showed an improvement in the color of the rumen epithelium (lighter color). Thus, this could be related to a better rumen health and consequently to a better VFA absorption through ruminal epithelium, reducing the content of total VFA but even more affecting VFA proportion, because propionic acid is quickly absorbed by simple diffusion (Allen et al., 2009; Allen and Bradford, 2012). Therefore, based on this research, our studies do not allow to completely confirm neither

reject propionic acid key role in the eating pattern modulation when citrus flavonoids are supplemented to high-concentrate fed bulls, and more research should be carried out to properly describe this possible mode of action.

Table 3. Ruminal VFA concentration and pH at slaughterhouse of Holstein bulls fed high-concentrate diets supplemented with citrus flavonoids.

	Pellet - Chapter IV		Meal - Chapter V		Fat - Chapter VI	
	Control	BF	Control	BF	Control	BF
pH	5.91	5.90			6.06	**6.57**
Total VFA, mM	111.10	121.70	75.60	67.20	131.30	**96.10**
Individual VFA, mol/100 mol						
Acetate	53.10	53.10	58.80	**66.40**	53.60	**58.90**
Propionate	35.90	36.20	28.90	**20.70**	35.90	**29.80**
Isobutyrate	0.70	0.60	7.40	7.50	0.80	0.90
n-butyrate	7.00	7.10	1.20	1.50	6.60	**7.20**
IsoValerate	1.50	**0.90**	1.50	1.30	1.7	1.70
Valerate	1.90	2.00	2.10	2.50	1.50	1.60
Acetate:propionate, mol/mol	1.60	1.60	2.15	**3.35**	1.60	2.10

- **Highlighted in bold**: statistical differences between treatments within the same study.
- ***Highlighted in bold and italics***: statistical tendency, between treatments within the same study.

On the other hand, also the importance of the ruminal fermentation on naringin metabolism has been discussed in our studies. As explained, naringin is the main citrus flavonoid in the pure extract of *Citrus aurantium* used in our studies. This glycosylated flavanone is the responsible of the bitter taste in so many citrus fruits, but this flavonoid is deglycosylated by ruminal flora, as *Butyrvibrio* spp, in naringenin (Simpson et al., 1969; Cheng et al., 1971). Thus, naringenin is completely the opposite of naringin from the taste point of view, acting as a potent bitter masking molecule. That could be the key point of these citrus flavonoids, because changes in ruminal fermentation could determine the effects observed when these flavonoids are supplemented. Thus, concentrate presentation (pellet vs. meal), concentrate composition (as fat level), or even the space feeder, that modulate the eating pattern of the animals and also ruminal fermentation, might affect naringin ruminal metabolism and its effects in bulls as well. All these factors could help us to explain the differences observed in the gene expression results in ruminal epithelium between bulls supplemented or not with citrus flavonoids, but also between

bulls that were supplemented with citrus flavonoids when the concentrate was fed in meal form, in pellet form or high fat content was added.

As far as we know, this is the first time that *TAS2R* have been studied in ruminal and duodenum epithelium of cattle. The possibility of the existence of these *TAS2R* in the digestive tract of cattle, and their possible role in the eating pattern modulation that had been observed in our first study was elucidated. Last decades, the study of the taste receptors located out of the oral cavity has been very important, and the importance of nutrient sensing throughout the digestive tract and its possible role in the gut-brain axis are being widely studied in humans and other mammal species. Numerous studies in different species are highlighting the implication of *TAS2R* in gastric emptying, satiety and gut motility (Glendinning et al., 2008; Janssen et al., 2011; Depoortere, 2014; Roura and Foster, 2018). In fact, *TAS2R* activation has been related with the release of anorexigenic hormones and peptides in the digestive tract, as cholecystokinin (*cck*), neuropeptide Y (*npy*), and peptide YY (*pyy*) (Chen et al., 2006; Depoortere, 2013; Takai et al., 2016). This anorexigenic response would be explained as an adaptive response to possible harmful and toxic molecules, and might explain the relationship between citrus flavonoids supplementation and the reduction observed in concentrate intake and meal size in bulls.

The gene expression of the different *TAS2R* studied (*TAS2R7*, *TAS2R16*, *TAS2R38* and *TAS2R39*) in the rumen epithelium were clearly affected by citrus flavonoids supplementation when concentrate was fed in meal form (Chapter V and VI), and this effect was lower when feeding the concentrate in pellet form (Chapter V) (**Table 4**). Actually, this observation would be in agreement with performance parameters obtained, as the results of the study performed with concentrate in pellet form (Chapter IV) showed no differences between treatments in concentrate intake, whilst a numerical reduction was obtained when concentrate was fed in meal form (Chapter V and VI).

Regarding anorexigenic hormones and peptides receptors (*cckbr* and *ppyr*), their gene expression were in concordance with *TAS2R* gene expression, either in rumen or in duodenum, independently of the study. When *TAS2R* were greater expressed, *cckbr* and *ppyr* were greater expressed as well (rumen epithelium, Chapter VI), and accordingly, when *TAS2R* were lesser expressed these anorexigenic receptors were also lesser expressed (rumen epithelium in Chapter V, and duodenum epithelium in Chapter VI)

(**Table 4**). Thus, these results would be in agreement with the fact that *TAS2R* receptors might be triggering an anorexigenic response when activated. Furthermore, factors affecting the eating rate (as concentrate presentation), and ruminal fermentation (as fat level and also pelleting the concentrate) would also affect the gene expression results of *TAS2R* in ruminal epithelium, and may be naringin and the ruminal metabolism of naringin could be modified by those dietary factors affecting the responses observed.

Table 4. Gene expression in ruminal and duodenum epithelium of the different studies.

	Rumen epithelium Pellet - Chapter IV		Rumen epithelium Meal - Chapter V		Rumen epithelium Fat - Chapter VI		Duodenum epithelium Fat - Chapter VI	
	CONTROL	BF	CONTROL	BF	CONTROL	BF	CONTROL	BF
TAS2R7	0.43	0.71	0.94	**0.13**	0.98	**8.52**	1.23	**0.11**
TAS2R16	0.46	***0.74***	0.94	**0.46**	0.91	**8.97**	0.98	**0.08**
TAS2R38	0.93	0.43	1.06	**0.38**	1.02	35.60	1.29	1.03
TAS2R39	0.53	0.81	0.88	**0.37**	1.03	**11.51**	1.10	**0.10**
ffar3	0.74	0.68	0.98	***0.64***	1.07	**13.89**	0.79	0.62
ffar2	0.40	0.64	0.96	**0.38**	1.06	**14.03**	0.98	**0.21**
adra2c	0.60	0.95	1.30	0.92	1.11	**41.13**	0.72	***0.24***
PPYR1	0.55	**0.80**	1.02	**0.55**	0.91	**10.14**	1.37	**0.28**
cckbr	0.55	0.69	1.04	**0.62**	1.09	**23.21**	1.01	**0.22**
Claudin	0.99	0.91	1.00	1.06	1.00	***0.86***	0.75	0.39
IL-25	0.44	***0.69***	0.98	**0.50**	1.01	**14.38**	1.00	**0.13**
TNFα	1.00	**1.67**	0.92	0.93	1.01	1.23	1.06	1.19
TLR4	0.88	0.92	0.97	**0.64**	1.00	**5.23**	0.94	**0.14**
Defensin-ß	0.52	***0.81***	1.00	**0.48**	1.01	**16.89**	1.00	**0.23**
Lactoferrin	1.00	1.02	0.97	1.00	1.02	2.63	1.05	1.00

- **Highlighted in bold**: statistical differences between treatments within the same study.
- ***Highlighted in bold and italics***: statistical tendency, between treatments within the same study.

In general, in Chapter IV, when concentrate was fed in pellet form in multi-space feeder, all the results obtained by supplementing citrus flavonoids were less pronounced. Actually, ruminal VFA and pH were practically not affected (**Table 3**), and the expression of genes at rumen epithelium was slightly affected as well (**Table 4**) when compared with the results of Chapter V and VI. Furthermore, in agreement with these results, also performance parameters studied did not showed any effect, not even a numerical reduction of the concentrate intake, as in the rest of studies (Chapter III, V and VI).

Pelleting the concentrate increases starch gelatinization and reduces particle size, but the hardness of the pellet also affect accessibility of ruminal bacteria to the nutrients (Bertipaglia et al., 2010). Therefore, when concentrate supplemented with citrus flavonoids was fed in pellet form, it would probably have been affecting ruminal deglycosylation of naringin to naringenin by reducing the accessibility of the ruminal bacteria to the naringin contained into the pellet. Consequently, this process would have taken more time compared with concentrate fed in meal form, and probably slowing down naringenin synthesis. On the other hand, if the eating rate is higher for pellet compared with concentrate in meal form (Verdú et al., 2017), also greater quantity of concentrate in a shorter time would arrive to the rumen for fermentation. So, these factors might have limited the transformation in the rumen of naringin to naringenin. Finally, the question would be why in the Chapter III, using the single-space feeder and concentrate in pellet form, concentrate intake was numerically reduced during the growing phase and meal size was reduced during the finishing phase as well. The answer might be related with the physical limitation of the access to the concentrate feeder, reducing the number of meals per day of the animals and probably increasing the time between meals of the bulls. Furthermore, bulls supplemented with citrus flavonoids devoted more time to eat straw. Consequently, it could be hypothesized that the single-space feeder might be promoting ruminal fermentation and metabolism of naringin to naringenin compared with multi-space feeder. Unfortunately, in this first study samples for gene expression analysis were not analyzed, that would have been very useful to better understand these differences between both studies (Chapter III and IV).

Otherwise, ruminal gene expression of *TAS2R* when high-fat concentrate was fed (Chapter VI) showed a clear an important increase in bulls supplemented with citrus flavonoids compared with non-supplemented animals. Conversely, the gene expression of these *TAS2R* observed in duodenum epithelium in the same animals showed completely the opposite results, being quite similar to the results obtained for *TAS2R* in ruminal epithelium when concentrate was fed in meal form (Chapter V) (**Table 4**). This fact is truly interesting, as it could be hypothesized that fat was protecting naringin from ruminal metabolism (as discussed in Chapter VI), but based on the results observed in the duodenum of these bulls, naringin would have had to be previously metabolized just before arriving to the duodenum. The gene expression of *TAS2R* observed in the duodenum of this bulls showed that naringenin should be acting there, as *TAS2R* were

lesser expressed that in non-supplemented bulls. The metabolism of naringin to naringenin can only be performed by bacteria enzymes, and then this metabolic transformation would have been done before arriving or at the duodenum, based on gene expression results. One possibility is that this step would have had to be accomplished in rumen and, then, the response observed in the rumen epithelium regarding *TAS2R* could be related with the high-fat content of the concentrate. In fact, in Chapter VI the possible interaction between bitter and fat was mentioned (Sala et al., 2013), and that could explain the response in the gene expression observed in the rumen epithelium when high-fat (palm oil) concentrate was fed to bulls supplemented with citrus flavonoids. The second possibility is that naringin to naringenin deglycosylation would have been performed after ruminal fermentation, acting the fat of the concentrate as a coating agent in rumen. In fact, among other bacteria, genus *Butyrvibrio* has mainly been found in the mucosa and digesta of rumen and reticulum, but also in omasum, abomasum and duodenum (Mao et al., 2015). Thus, this deglycosylation of naringin to naringenin after ruminal fermentation and before arriving to duodenum or directly in the duodenum is completely possible.

The specific nutrient-sensing receptors for fats are the free fatty acid receptors (*ffar*). There are different and specific receptors activated by different fatty acids depending on the chain length (Mielenz, 2016). Thus, *ffar2* and *ffar3* are both activated by short chain fatty acids (Wang et al., 2009 and 2012; Hudson et al., 2012; Friedrichs, 2015), so VFA. Contrary to other species, that use carbohydrates as the most important energy source, VFAs are the main energy source for ruminants (Siciliano-Jones and Murphy, 1989). Therefore, VFAs would play an important role as cues for energy homeostasis and food intake modulation in cattle, being normal that their mRNA expression have been found in many tissues of cattle, and especially in the digestive tract (Wang et al., 2009 and 2012; Friedrichs, 2015; Devant et al., 2016; Mielenz, 2017). These *ffar*, as *TAS2R*, are been related with the secretion of different anorexigenic molecules such as *glucagon-like peptide-1* (*GLP-1*), *ppy* and *cck* (Damak, 2016; Mielenz, 2016). That would reinforce the hypothesis that it would have been generated some kind of interaction between bitter and fat response in the rumen epithelium, but much more research is needed to find a response, even more to explain the eating pattern modulation resulting from this interaction. Bitter and fat clearly trigger satiety and anorexigenic responses, so probably both would share metabolic pathways, and a hierarchy in the response between them might exist.

Lastly and most importantly, when analyzing the results obtained in the rumen epithelium gene expression of the different studies (Chapter IV, V and VI) and the expression of the same genes in the duodenum epithelium (Chapter VI) different questions appear. First of all, the eating pattern and concentrate intake results obtained in Chapter V and VI are quite similar, whilst the gene expression at rumen level was completely the opposite. Secondly, gene expression in the duodenum epithelium (Chapter VI) was closer to the gene expression results obtained in rumen epithelium in Chapter V. Although it would be very interesting and useful to have the gene expression results in duodenum epithelium of bulls of the Chapter V, it could be assumed that this gene expression should be very similar of that obtained in Chapter VI, as the metabolism of naringin into naringenin is irreversible. So, probably the questions that should be answered is which mechanisms are modulating the eating pattern of these bulls: i) nutrient-sensing mechanisms located in the rumen or duodenum epithelium, ii) both rumen and duodenum are interrelated, and finally iii) if it exists any relationship of hierarchy between rumen and duodenum nutrient-sensing mechanisms.

Actually, the results of these studies show that there are nutrient-sensing mechanisms in the digestive tract of bulls beyond ruminal fermentation byproducts (as VFA and propionic acid), acidosis (pH) and rumen microorganisms that would be affecting the eating pattern, and consequently performance. Furthermore, probably all these mechanisms are interrelated, and nutrient-sensing receptors located in the digestive tract are detecting these VFA, nutrients, pH and ruminal microflora byproducts, to properly maintain the required energy homeostasis, and regulating hunger and satiety in bulls.

Traditionally, the study of nutritional additives and also natural products, such as essential oils, tannins, saponins, and different natural extracts, have been focused on their effects on ruminal microflora, ruminal acidosis and ruminal fermentation modulation. Actually, an important part of their possible modes of action should be considered, as probably most of them and their ruminal metabolites might be able to interact directly with different nutrient-sensing receptors distributed along the digestive tract. Thus, future research probably should consider also nutrient-sensing mechanisms as an important part for developing and studying new feed additives, natural alternatives to improve cattle

efficiency and health, but even to properly adapt nutrition and diet to the digestive system of these animals.

2. ANIMAL BEHAVIOR MODUALTION IN BULLS BY CITRUS FLAVONOIDS

Animal welfare is an important pillar for our intensive animal production systems future, basically due to its value for consumers and public opinion, but also to its positive consequences on animal health and efficiency.

As discussed in the different chapters of this thesis, in beef cattle, aggressive behaviors and stereotypes are been related to frustration and discomfort, and would indicate poor welfare status (Gonyou, 1994; Devant et al., 2016). Citrus flavonoids supplementation have reduced oral non-nutritive behaviors, aggressive and sexual interactions in bulls fed high-concentrate diets and reared under intensive commercial conditions (Chapter III, IV, V and VI), independently of the feeder space, the concentrate presentation (pellet vs. meal) or composition (fat level).

Along the different chapters, various hypothesis have been postulated to explain the possible mechanisms whereby these citrus flavonoids would be modulating the animal behavior in these bulls, such as the gut-brain axis mechanisms, rumen health, and also the eating pattern modulation, as the possible relationship with time to perform feeding events.

3.1. Gut-brain axis mechanisms

As previously explained, the gut-brain axis is a communication network between the digestive system and the brain. Furthermore, it has been proposed as an important cross-talk mechanism involved in mood modulation and behavior in humans and other animals (Evans et al., 2013; Haagensen et al., 2014; Wiley et al., 2017). In fact, gut-brain axis would be probably one of the most complex systems involving nutrient sensing mechanisms, and also the immune and nervous system, to connect digestive tract, microflora and brain. Thus, inflammation has been proposed as a key player into this network, basically by reducing serum serotonin concentration, an important neurotransmitter associated with mood modulation as well (Evans et al., 2013).

Devant et al. (2016) after analyzing the expression of some genes related with gut-brain axis in ruminal epithelium of beef cattle fed high-concentrate diets suggested that in cattle some of the mechanisms of this biologic cross-talk network could take place in rumen. Thus, after the modulation of bulls' behavior observed in our first study (Chapter III), and considering the well-known anti-inflammatory properties of citrus flavonoids, the hypothesis that citrus flavonoids supplementation might modulate animal behavior through mechanisms involved in the gut-brain crosstalk was considered. Nutrient sensing mechanisms (as *TAS2R* and *ffar*), molecules regulating gastrointestinal inflammation (such as *IL-25*, *TNFα*, *TLR-4*, *ß-defensin*, and *lactoferrin*) and neuropeptides could play a relevant role in this animal behavior modulation through the gut-brain axis cross-talk.

Along the different chapters, the study of the gene expression in rumen (Chapter IV, V and VI) and duodenum epithelium (Chapter VI) has showed important differences when citrus flavonoids were supplemented. Additionally, ruminal gene expression differed among the studies, so the gene expression of genes involved in the modulation of behavior when bulls supplemented with citrus flavonoids were different depending on the concentrate presentation (pellet or meal; Chapter IV and V), and composition (fat level inclusion; Chapter VI) (**Table 4**). Whereas the gene expression of proteins related with the inflammation in the rumen epithelium in Chapter V (meal) were reduced with citrus flavonoids supplementation, in Chapter IV (pellet) and VI (fat) the gene expression of pro-inflammatory molecules were increased (Chapter IV) or, even more, markedly increased in the case of high-fat diets. The gene expression of these proteins related to inflammation, as observed for anorexigenic peptides receptors (*cckbr* and *ppyr*), were again in concordance with *TAS2R* gene expression throughout the different studies (**Table 4**).

In our studies, the gene expression has analyzed mRNA in the epithelium of rumen and duodenum of the animals, however where these *TAS2R* are located in the epithelium has not been studied. Tuft cells are solitary chemosensory cells that has been described in different parts of the gastrointestinal (stomach and intestine) and respiratory tract (Breer et al., 2012). These tuft cells have been proposed to act as immune sentinels in the intestinal lumen (Ting and Moltke, 2019), acting as key link to different infections (such as viruses, protozoa, and helminthes) and also alterations in the gut microflora (Moltke et al., 2016; Lou et al., 2019). Recently, Lou et al. (2019) have demonstrated that tuft cells

in different parts of the intestine in rats are activated by different *TAS2R*, and directly inducing the release of *IL-25*. These results would suppose a direct link between *TAS2R* and immune response, and would be in agreement with the results obtained in gene expression studies carried out in rumen and duodenum epithelium, as *IL-25* is greater or lesser expressed in concordance with gene expression of *TAS2R* (as other proteins related to inflammation analyzed, such as *TLR-4*, and *defensin*).

On the other hand, the possible link between gene expression results of the different studies and the improvement in animal behavior observed, reducing oral non-nutritive behaviors, and also aggressive and sexual interactions, is not so clear. The gene expression results obtained in rumen epithelium clearly differs between Chapters IV, V and VI, so probably nutrient sensing and inflammation in rumen are closely related with ruminal fermentation and metabolism of citrus flavonoids (especially deglycosylation of naringin to naringenin) and fat level, being clearly affected by diet.

Actually, with the available data is not possible to conclude how these genes analyzed in ruminal and duodenal epithelium are or not determinant in the animal behavior regulation observed in our studies. Three hypothesis could be contemplated: i) these genes are not determinant in the response observed in our studies; ii) as in other mammals, maybe these mechanisms are important at gut level, but rumen is not involved; or iii) deeper research is needed, as the study of gene expression performed is limited, because only mRNA of epithelium is analyzed. Cellular manifestations, molecular differences may be exhibited across multiple layers of gene regulation like genomic variations, gene expression, protein translations and post-translational modifications. This needs the integrative analyses to understand the interplay of the individual components of the biological system; in the present thesis only a small part (mRNA transcript of some genes) of the whole molecular mechanisms has been described.

3.2. Oral non-nutritive behaviors and rumen health

Oral non-nutritive behaviors, as licking objects with non-nutritional finality, is an abnormal behavior frequently performed by beef cattle reared under intensive production systems (Bergeron et al., 2006). In beef cattle, as a ruminant, performing eating behaviors such as foraging or ruminating are fundamental, and not always properly satisfied under

intensive conditions. Consequently, digestive dysfunctions such as rumen lesions, low ruminal pH, or low rumination activity have been related with the occurrence of this non-nutritive behaviors (Bergeron et al., 2006; Devant et al., 2016).

Supplementing concentrate with citrus flavonoids have clearly and constantly reduced the performance of this oral behaviors in bulls throughout the different studies, independently of the conditions (feeder design, concentrate presentation or composition) (**Table 1**). In the different studies, bulls supplemented with citrus flavonoids devoted more time to ruminating activities (**Table 1**), with statistical differences (Chapter IV, V and VI) or only numerical (Chapter III; but in this study using the single-space feeder these bulls devoted more time to eat straw). Furthermore, in all cases, the macroscopical study of the rumen wall performed at the slaughterhouse showed lighter color for bulls supplemented with these citrus flavonoids (**Table 5**). Actually, darker color of the rumen wall have been related to inflammation due to iron deposition in some studies carried out in our research group (data not published). Surprisingly, regarding baldness areas in the rumen wall, results varied between the different studies: were reduced when citrus flavonoids were supplemented in Chapter III, but increased in Chapter V, whilst no effect was observed for the remain studies (Chapter IV and VI). In fact, although we do not have an explanation for this result, that might be related with the different ruminal fermentation and eating pattern of the bulls depending on the concentrate presentation (pellet vs. meal), the feeder design and concentrate compositon.

On the other hand, citrus flavonoids, and especially naringenin, possess potent antioxidant and anti-inflammatory properties (Manchope et al., 2017), so could be also improving rumen wall of the bulls supplemented. Furthermore, in the Chapter VI, bulls supplemented with citrus flavonoids also had lesser ruminal pH, and VFA content was lesser as well (**Table 3**).

Thus, it could be hypothesized that citrus flavonoids might reduce oral non-nutritive behaviors increasing ruminating activity of bulls, and improving rumen health as well.

Table 5. Rumen health parameters macroscopically studied of the different studies.

	Single-space feeder Chapter III		Pellet Chapter IV		Meal Chapter V		Fat Chapter VI	
	Control	BF	Control	BF	Control	BF	Control	BF
Color of the rumen								
1					45.71	**63.01**	9.59	**22.22**
2			2.67	**2.86**			36.99	**50.00**
3	42.68	***44.30***	48.00	**70.00**			53.42	**26.39**
4	47.56	***54.44***	48.00	**24.29**	54.29	**36.99**	-	**1.39**
5	9.76	***1.27***	1.33	**2.86**				
Papillae clumping								
Yes	43.90	40.51	40.00	44.29	22.86	20.55	16.44	***6.94***
No	56.16	59.49	60.00	55.71	77.14	79.45	83.56	***93.06***
Baldness region								
Yes	67.07	**48.10**	40.00	49.33	38.57	**58.90**	39.73	48.61
No	32.93	**51.90**	60.00	50.67	61.43	**41.10**	60.27	51.39

- **Highlighted in bold**: statistical differences between treatments within the same study.
- ***Highlighted in bold and italics***: statistical tendency, between treatments within the same study.

3.3. Time devoted to eat and animal behavior

Generally speaking, when bulls were supplemented with citrus flavonoids dedicated more time to eat concentrate and performed greater ruminating activity. That could lead to a reduction in aggressive and sexual interactions observed during the visual scan procedure.

Some nutritional strategies intended to increase time devoted to perform eating behaviors (as reducing energy content of the diet by increasing fiber content), reduced aggressive and abnormal behaviors in broilers (Qaisrarni et al., 2012). Basically, that could be explained because animals devoting more time to eat devoted less time to perform other behaviors, as aggressive and sexual interactions in these bulls.

On the other hand, Lindström et al. (2000) found that eating events and feed oral manipulation in cows were linked to greater oxytocin levels in blood independently of the rumen feed content. Furthermore, this was related with lower cortisol levels in blood plasma, and lower stereotypes were observed in the animals. Consequently, this study would demonstrate the importance of feeding events, eating or ruminating, in cattle, and their direct link with animal behavior. In agreement with this study, citrus flavonoids supplementation might improve animal behavior and welfare by increasing time devoted

to eat concentrate, straw or ruminating activity. In a future research would be also very useful to analyze oxytocin or cortisol levels in blood of bulls supplemented with citrus flavonoids.

2. FUTURE RESEARCH

The last 20 years there have been an important number of reports published describing the presence of taste and odorant receptors in non-oral and non-olfactory tissues of different vertebrate species. Regarding taste receptors, they have been found in a wide range of tissues and organs out of the mouth, like gastrointestinal and respiratory tract, brain, skeletal muscle, and testis in different species (Kiuchi et al., 2006; Behrens and Meyerhof, 2011; Beer et al., 2012; Colombo et al., 2012; Foster et al., 2014; Roura and Foster, 2018). The functions of these taste receptors are being deeply studied at the present and, within the digestive tract, they seem to be involved in important and complex functions in the communication pathways between the gastrointestinal system and the brain. Consequently, they might be involved in the gut-brain axis acting as a sensors for food-derived components, being key players in the hungry and satiety mechanisms through the release of hormones which could act locally (in a paracrine way) or systematically (by circulatory or lymphatic systems) (Breer et al., 2012; Roura and Foster, 2018).

In regards to cattle, there is scarce literature focused on nutrient sensing or describing the presence of these taste receptors, either in the digestive tract or in other tissues. Although *TAS2R* functions in gastrointestinal tract of cattle have not been profoundly explored, the modulation of the eating pattern along with the modification in gene expression of *TAS2R* in rumen and duodenum epithelium found in our studies might be consistent with the effects observed in other species.

Nowadays, secondary plant metabolites as polyphenols, essential oils, and different natural extracts are being proposed as referred to as natural alternatives to improve animal health, performance and animal behavior. Futhtermore, when talking about natural porducts in ruminants, it is also quite relevant to take into account their possible metabolites after ruminal fementation. These metabolites could be playing important roles in the mode of action, in some cases, they could be even more important than the original molecule used.

The mechanisms whereby these non-nutrient compounds and phytochemicals are actually acting are not well-known and described. In fact, many of these non-nutrient compounds are considered to trigger biter taste, so probably *TAS2R* are playing a key role in the effects we observed when these type of molecules are used. As discussed throughout this thesis, *TAS2R* seem to be involved in so many important functions, as hunger and satiety regulation (through anorexigenic molecules regulation), and immune response modulation (by inflammation and innate immune response). Consequently, if these natural molecules aim to be real alternatives for the future in cattle, *TAS2R* should be deeper investigated, so a deeper knowledge about their metabolic functions, how they are activated or blocked, or where they are located.

Actually, a better knowledge about all the mechanisms involved in the gut-brain axis will be essential to understand and develop useful natural products that could improve animal health, efficiency and welfare.

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CHAPTER VIII

Final Conclusions

FINAL CONCLUSIONS

The different studies performed in this thesis to study the effects of supplementing citrus flavonoids extracted from *Citrus aurantium* on Holstein bulls fed high-concentrate diets under commercial conditions, have allowed to achieve the following conclusions:

1. Citrus flavonoids supplementation modified eating pattern of bulls, reducing the percentage of large meal sizes in single-space feeders and increasing the time devoted to feeding behaviors independently of the feeder space, concentrate presentation (pellet and meal) and composition (fat level inclusion).

2. When the single-space feeder was used in bulls supplemented with citrus flavonoids concentrate intake was reduced impairing final body weight, although these differences disappeared in the hot carcass weight. Consequently, feeder space shoud be considered when adding citrus flavonoids because it coud limit the access to the concentrate feeder.

3. The effects of citrus flavonoids on concentrate intake were affected by concentrate presentation form, pellet or meal. When concentrate presentation was meal, concentrate intake (especially during the growing phase) was reduced when supplementing citrus flavonoids without impairing performance. Consequently, these bulls tended to be more efficient compared with non-supplemented bulls under our farm conditions. On the other hand, when concentrate presentation form was pellet, citrus flavonoids supplementation did not affect intake or performance.

4. During the finishing phase, in bulls fed high-fat (palm oil) concentrate citrus flavonoids supplementation did not have any synergic effect reducing concentrate intake, however time devote to eat concentrate and ruminating activities during the visual scan procedure was increased.

5. Rumen wall color macroscopically studied at the slaughterhouse was lighter for bulls supplemented with citrus flavonoids compared with control animals throughout the different studies, independently of the feeder space, presentation (pellet and meal) or fat level of the concentrate fed. This could be related to better ruminal health, probably due to eating pattern modulation (lesser ruminal pH fluctuations), along with antioxidant and anti-inflammatory properties of the citrus flavonoids.

6. Animal behavior was positively affected by citrus flavonoids supplementation in the four studies performed: oral non-nutritive behavior, aggressive and sexual interactions of bulls were reduced. The modulation of these behaviours could be explained by the eating pattern modulation (more time to perform feeding behaviors), together with the improvement in rumen wall health parameters observed when citrus flavoids were supplemented.

7. Citrus flavonoids supplementation modified the expression of genes in the rumen epithelium related to the gut-brain axis cross-talk (nutrient sensing receptors, peptides and hormones receptors, and some pro-inflammatory molecules). The effect was different among the studies, so the expression of these genes involved in nutrient sensing and behavior in bulls supplemented with citrus flavonoids was different depending on the concentrate presentation (pellet or meal), and composition (fat level inclusion). Thus, suggesting a possible important effect of ruminal fermentation on flavonoid metabolism and, consequenly, on its effects. In bulls fed high-fat diets, in the duodenum epithelium the expression of these genes was also modified when supplementing citrus flavonoids.

8. The expression of *TAS2R* genes was clearly modified by citrus flavonoids supplementation in rumen and duodenum epithelium when concentrate was fed in meal form, athough these effects were lighter when concentrate was fed in pellet form. Moreover, the gene expression of receptors for anorexigenic peptides and hormones studied (*ppyr1*, *cckbr* and *adra2c*) was also affected by citrus flavonoids supplementation. This modulation was in concordance with the gene

expression results of *TAS2R* in the different tissues (rumen and duodenum), probably suggesting that *TAS2R* would be linked to anorexigenic molecules and response.

9. The gene expression of pro-inflamatory molecules analyzed, such as *IL-25*, *TLR-4*, and *β-defensin* was greater or lesser expressed also in concordance with the gene expression of *TAS2R* in ruminal and duodenal epithelium. These results would suggest a direct link between *TAS2R* and immune response as well.