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. 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 than in C bulls (5.11 vs. 5.36 ± 0.108 kg/kg). Percentage of animals eating concentrate during visual scan was greater ($P < 0.01$) in BF compared with C bulls (10.02 % vs. 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.

Abbreviations: ADG, average daily gain; ADF, acid detergent fiber; ANOVA, analysis of variance; BW, body weight; CP, crude protein; CV, covariance; DM, dry matter; EE, ether extract; FCR, feed conversion ratio; HCW, hot carcass weight; ME, metabolizable energy, NDF, neutral detergent fiber; NFC, non-fiber carbohydrates; SEM, standard error of the mean; TAS2R, bitter taste receptors; VFA, volatile fatty acids

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, Paniagua et al. (2018) studied in Holstein bulls fed high-concentrate diet the effect of an extract from bitter orange (*Citrus aurantium*) rich in naringin during the growing and finishing phase. 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 (Paniagua et al., 2018). 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%; BF) (Bioflavex CA, Interquim, S.A., Barcelona,

Spain). Bioflavex was incorporated with the same procedure as premix was added to 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.

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 167 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**.

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. 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 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 (*GPR43*) and free fatty acid receptor 3 (*GPR41*), 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 3**). 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 3**). 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,

267 autoregressive order one, and unstructured. The covariance structure that yielded the
268 smallest Schwarz's Bayesian information criterion was considered the most desirable
269 analysis.

270 In the case of rumen gene expression and VFA data pen were considered the experimental
271 unit and animals as sampling units, and data were analyzed using ANOVA where the
272 model included treatment (as there were no repeated measures) as the main effect. For
273 categorical variables analyses (carcass classification, rumen health parameters, hepatic
274 abscesses) a Chi-square-test was used.Differences were declared significant at $P < 0.05$,
275 and trends were discussed at $0.05 \leq P \leq 0.10$ for all models.

276 **3. Results**

277 *3.1. Animal health*

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

281 *3.2. Intake, performance and carcass quality*

282 No statistical differences were found in concentrate intake between treatments, neither
283 during the growing phase (**Table 4**) nor for the finishing phase (**Table 5**). Also for the
284 whole study (**Table 6**), no differences between treatments were observe for this
285 parameter. In the same way, estimated straw consumption did not show statistical
286 differences during the growing phase ($P = 0.92$) (0.57 vs, 0.56 ± 0.046 kg/d for C and BF,
287 respectively) nor for the finishing phase ($P = 0.46$) (0. 89 vs. 0.97 ± 0.074 kg/d for C and
288 BF, respectively) (data not presented). Throughout the study straw consumption
289 estimated were 0. 73 ± 0.080 kg/d for C group and 0.77 ± 0.080 kg/d for BF animals, and
290 no statistical differences were observed either ($P = 0.74$) (data not presented).

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 7**. At the slaughterhouse BW, dressing percentage, carcass conformation and fatness classification were not affected by treatment.

3.3. Animal behavior

General activities. All data for animal behavior, general activities and active behavior as well, are showed in **Table 8** and **Table 9** for growing and finishing phase, respectively. 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.

315 *Active behavior*. In the growing phase, during the visual scan observation period, the only
316 parameter not affected by treatment was the social behavior. Self-grooming behavior was
317 greater for BF compared with C bulls, and C bulls exhibited more ($P < 0.01$) oral non-
318 nutritive behaviors than BF bulls. BF bulls exhibited less ($P < 0.01$) agonistic interactions
319 than C bulls. Fighting, displacement, chasing and chasing-up, and butting behaviors were
320 greater ($P < 0.05$) in C than in BF bulls. Flehmen behavior was greater ($P < 0.05$) in C
321 compared with BF bulls. Additionally, attempt to mount and complete mounts tended (P
322 < 0.10) to be greater in C than BF bulls.

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

328 *3.4. Macroscopic rumen evaluation and liver abscesses*

329 At the slaughterhouse (**Table 10**), color of rumen wall was lighter ($P < 0.05$) for BF bulls
330 (63.01% classified as color < "3") compared with C bulls (45.71 classified as color <
331 "3"). Baldness areas presence in the rumen were greater ($P < 0.05$) in BF bulls (58.90%)
332 than in C bulls (38.57%). No differences between treatments were observed in the
333 remaining macroscopic parameters analyzed at the slaughterhouse (liver abscesses, ulcers
334 and clumped papillae).

335 *3.5. Rumen VFA concentration at slaughterhouse*

336 Rumen VFA concentration data are presented in **Table 11.** Total VFA concentration in
337 the rumen was not affected by treatment. The molar proportion of acetate was greater (P
338 < 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.

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 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. Paniagua et al. (2018) found a reduction in concentrate intake explained by a numerical decrease of meal size 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

363 occupancy tended to be greater for BF animals. This supports the hypothesis that these
364 animals were able to compensate meal size reduction by increasing the number of visits
365 to the feeder. Additionally, in the present study BF bulls showed higher ADG than C bulls
366 during this finishing phase without an increase of concentrate intake. Consequently, BF
367 bulls were more efficient during this phase, although concentrate efficiency improvement
368 was only numerical. Smaller meal sizes have been related to an improvement of feed
369 efficiency in steers (Montanholi et al., 2010). Then, assuming that bulls supplemented
370 with flavonoids may have reduced the large meal sizes (> 750 g/ meal) (Paniagua et al.,
371 2018), this could explain the improvement in concentrate efficiency during the finishing
372 phase and the greater ADG of these BF animals. In a previous *in vitro* study Seradj et al.
373 (2014) observed that BF decreased methane production which could explain the
374 efficiency improvement in BF supplemented bulls observed in the present study. Another
375 possible mechanism involved in the feed efficiency improvement in beef animals
376 described in the literature is based on the rumen VFA profile. Greater propionate
377 percentage and lesser acetate:propionate ratio in rumen fluid are related with better
378 efficiency in ruminants when ionophore antibiotics are added to the feed (Golder and
379 Lean, 2016). Previously, in the study carried out by Balcells et al. (2012), an increase in
380 molar proportions of propionic acid in the rumen of cannulated heifers supplemented with
381 citrus flavonoids was observed. Propionic acid has been described as an important
382 regulator of feed intake in ruminants fed high starch diets (Bradford and Allen, 2007).
383 According to these results, Paniagua et al. (2018) hypothesized that supplementation with
384 citrus flavonoids could reduce large meal sizes in bulls due to an increase in ruminal
385 propionate production. However, the results of the current study showed greater rumen
386 molar percentage of propionate at slaughterhouse in C bulls than in animals supplemented
387 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 the results obtained by Paniagua et al. (2018), in the present study the color of the rumen wall was lighter for BF bulls. 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).

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

438 naringin to naringenin (Simpson et al., 1969; Cheng et al., 1971) as well. Contrary to
439 naringin, naringenin acts as an important bitter masking molecule (Jacob et al., 2014).
440 Roland et al. (2014) described how some flavanones act as actual antagonists for human
441 *TAS2R39*, reducing the receptor response possibly by orthosteric mechanism acting over
442 a single binding pocket of the bitter taste receptor. Accordingly, our results would agree
443 with the function of naringenin as a bitter masking molecule, acting as antagonist for all
444 TAS2R analyzed. This reduction in the gene expression of TAS2R could be related with
445 the greater time devoted to eat observed in BF bulls. Actually, our results have also
446 showed a clear decline in the gene expression of receptors related with neurotransmitters,
447 as *cckbr* (acts as *CCK* and *gastrin* receptor; Silvente-Poirot and Wank, 1996) and *ppyr1*
448 (acts as *NPY* and *PYY* receptor; Larhammar, 1996) in bulls supplemented with flavonoids,
449 that would be in concordance with the reduction in the expression of the TAS2R analyzed
450 in the rumen epithelium of these animals, because of these taste receptors are involved in
451 the release of these anorexigenic molecules. Consequently, we can hypothesize that
452 supplementation with citrus flavonoids in bulls might modulate eating pattern acting over
453 TAS2R expressed in the rumen epithelium, and consequently modifying the release of
454 hormones and bioactive peptides involved in hunger and satiety. Therefore, some citrus
455 flavonoids or their derivates might act as bitter masking molecules in the rumen, and bulls
456 supplemented with these flavonoids devoted more time to eat, and this could be related
457 to a decrease in agonist and sexual interactions.

458 Although reducing meal size and increasing occupancy of the feeder at the same time
459 could be considered a contradiction, in fact, naringin supplemented in the concentrate is
460 a bitter molecule that would be triggering the release of anorexigenic hormones and
461 peptides, until is metabolized into naringenin. Conversely, naringenin would be acting as
462 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, 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. Thus, 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, Paniagua et al. (2018) observed in bulls supplemented with the same citrus flavonoids a reduction of oral non-nutritive behaviors, and aggressive and sexual interactions. 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.

As previously observed (Paniagua et al., 2018), 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 *IL-25, TLR4*, and *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). *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 (Paniagua et al., 2018), 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

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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762 **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
NDF, g/ kg DM	178	151
NFC, g/ kg DM	550	628

763 [1] from 0 to 112 days of the study.
764 [2] from 113 days to the end of the study.
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773 **Table 2.** Description of the social behavioral categories recorded.

Interactions	Item	Definition
Nonagonistic interactions	Self-grooming	Nonstereotyped 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 push vigorously head against head.
	Butting	When one bull pushes vigorously its head against any part of another bull's body.
	Displacement	When one bull jostles itself between 2 other bulls or between a bull and any equipment.
	Chasing	When a bull follows fast or run behind another bull.
	Chasing-up	When a bull pushes 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 stereotypies	Tongue rolling, stereotyped licking or bitting any equipment

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776 **Table 3**. Sequence, annealing temperature (At), concentration (μM), efficiency (%) and

777 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

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

	Treatment[1]			*P*-value[2]		
Item	Control	BF	SEM3	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
Water intake						
Mean, kg/d	17.93	19.87	1.683	0.42	<.0001	0.28
CV, %	31.43	24.01	7.255	0.47	0.36	0.40
FCR, kg/kg	4.50	4.34	0.087	0.19	<.0001	0.22

782 [1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
783 [2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by
784 time interaction effect.
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Table 5. 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
Water intake						
Mean, kg/d	28.35	31.04	1.500	0.22	<.0001	0.01
CV, %	42.05	16.85	8.351	<0.05	0.58	0.54
FCR, kg/kg	7.08	6.66	0.297	0.34	<.0001	0.049

[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 6. 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
Water intake						
Mean, kg/d	21.42	23.58	1.411	0.28	<.0001	0.07
CV, %	34.77	21.30	6.992	0.18	0.61	0.51
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 7. 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.

860 **Table 8**. General activities (%) and social behavior (times/ 15 min) during the growing
861 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

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

863 2. T = treatment effect; Time = time effect (measurements every 14 d); T x Time =
864 treatment by time interaction.
865 3. SEM = standard error of the means of the log-transformed data (general activity) or
866 root transformed data (social behavior).
867

868 **Table 9**. General activities (%) and social behavior (times/ 15 min) during the finishing
869 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

870 1. C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
871 2. T = treatment effect; Time = time effect (measurements every 14 d); T x Time =
872 treatment by time interaction.
873 3. SEM = standard error of the means of the log-transformed data (general activity) or
874 root transformed data (social behavior).
875

Table 10. 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	

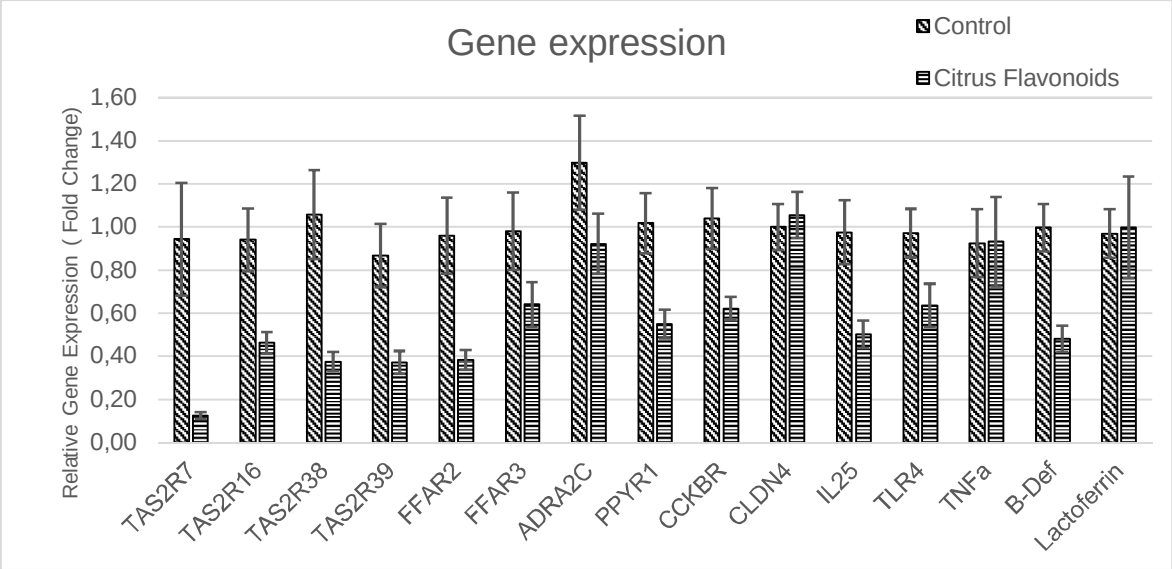
[1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
[2] T = treatment effect.
[3]Adapted from Gonzalez et al. (2001): Rumen color: 1= white; 5 = black.
[4]Adapted from Nocek et al. (1984).

884 **Table 11.** Rumen VFA concentration at slaughterhouse of Holstein bulls fed high-
885 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

886 [1] C = non-supplemented, BF = concentrate supplemented with citrus flavonoids.
887 [2] T = treatment effect.
888

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*
IL-25: *Interleukin-25*
TLR4: *Pattern recognition receptors, like Toll-like receptor 4*
TNFa: *Tumor necrosis factor alpha*
B-Def: *Beta-defensin*