FLAVONOIDS AND RUMEN HEALTH AND PERFORMANCE

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 one of 6 pens and 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 exhibited more displacements and fighting than C bulls, whilst C bulls performed more ($P < 0.05$) oral behaviors. 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

Bioflavex® CA, feed intake was reduced. Flavonoids 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** maybe due to the reduction of large meal sizes.

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

INTRODUCTION

Flavonoids are widely distributed in the plant kingdom, i.e. in fruits, seeds, vegetables, tea, 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 lactatic 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-

72 starch diets, affecting both satiety and hunger (Oba et al., 2002), the supplementation of
73 flavonoids could affect eating pattern of bulls fed high-concentrate diets. Moreover, this
74 supplementation could reduce methane production, and together with the reduced
75 ruminal pH fluctuations (Lam, 2016) could increase efficiency of nutrient utilization in
76 steers.

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

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

90 **MATERIALS AND METHODS**

91 **Animals, Feeding, Housing, and Experimental Design**

92 The study was conducted in accordance with the Spanish guidelines for experimental
93 animal protection (Royal Decree 53/2013 of February 1st on the protection of animals
94 used for experimentation or other scientific purposes; Boletín Oficial del Estado, 2013).
95 Animals were fattened under commercial conditions in a farm (Agropecuaria Montgai

96 SL, Montgai, Lleida). One hundred ninety-eight Holstein bulls (195.3 ± **19.6** kg of **body**
97 **weight (BW)** and 149 ± **6.8** d of age) in two consecutive fattening cycles (99 animals
98 each cycle) were used.

99 Animals were randomly allocated in one of six covered pens (12 m long x 6 m wide)
100 that were deep-bedded with straw and equipped with a computerized concentrate single-
101 space feeder (0.50 m long x 0.26 m wide x 0.15 m depth) with 10 kg of concentrate
102 capacity as described elsewhere (Verdú et al, 2015), with lateral protections (1.40 m
103 long x 0.80 m high) forming a chute, which width could be adapted from 42 to 72 cm,
104 depending on the animal size and age (Verdú et al., 2015). This computerized feeding
105 system was calibrated weekly (Verdú et al., 2017). When each animal visited the feeder,
106 it was identified, the computer recorded the initial and final concentrate's weight, with
107 its initial and final time. Animals were adapted during 3 wk by widening the chute to
108 facilitate feeder access (adaptation period). During the study, the width of the chute has
109 been adapted to the animal size to allow them to eat easily.

110 Pens were also equipped with a water bowl and a separated straw feeder (3.00 m long x
111 1.12 m wide x 0.65 m depth; 7 feeding spaces) where straw was offered ad libitum.

112 **Feed Intake and Performance**

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

The study design 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**.

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)**

144 **using sodium sulfite and alpha-amylase**, and EE by Soxhlet with a previous acid
145 hydrolysis (method 920.39; AOAC, 1995).

146 Naringin was determined for every sample as a Biofalvex® CA marker for BF group,
147 and was used as a quality control analysis to guarantee the correct addition of the
148 product into the feed by Laboratory of Interquim S.A. Internal method for naringin
149 quantification using HLPC developed by Interquim S.A. was used **and analyzed as**
150 **described herein. To analyze naringin all** concentrate samples were milled. Five
151 grams were weighed and 50 mililiters of dimethyl sulfoxide were added and agitated for
152 15 min, and was filtered and placed in a vial. The pattern was prepared, 30 mg of
153 naringin were mixed with dimethyl sulfoxide until 100 ml were achieved. Drying losses
154 were taken into account for calculations. Nova-Pak C18 columns were used as
155 stationary phase for the chromatography, silica-based, reversed-phase C18 columns that
156 are based on 4 μm particle technology (Waters Cromatografia SA, Cerdanyola del
157 Vallés, Barcelona). The column was maintained at 40ºC, acidified water with methanol
158 R (70:30) v/v was used as mobile phase, with a flow rate of 1.0 mL/min. 10 μL were
159 injected, and detection was done by UV at 284 nm. The chromatography duration was
160 around 35 min.

161 **Animal Behavior**

162 A visual scan procedure at days 16, 31, 44, 59, 72, 87, 100, 114, 128, 142, 157, and 168
163 of the study was performed to study the general activity (standing, lying, eating,
164 drinking, and ruminating) and social behavior (nonagonistic, agonistic, and sexual
165 interactions) of the animals in every pen. Social behavior activities recorded are
166 described in **Table 2**. The visual observation was made for 2 pens at the same time from
167 8:00 to 10:00 **h,** as described by Mach et al. (2008), Rotger et al. (2006), Robles et al.
168 (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).

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.**

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).

Calculations and Statistical Analyses

193 Pen was considered the experimental unit and animals within pen were considered
194 **observational** units for all statistical analyses**. Two pens (one of the C group and one**
195 **of the BF group) belonged to the first fattening cycle were removed due to**
196 **technical problems with the antenna of the single-space feeder, and all data of**
197 **these animals were deleted from the databases.**

198 Meal criteria for each animal and period was calculated as described by Bach et al.
199 (2006). Thus, visits at the single-space feeder were separated into meals, and eating
200 pattern parameters (meal frequency, meal duration, inter-meal duration, and meal size)
201 were calculated. To calculate performance, eating behavior and concentrate
202 consumption, all individual data registered were averaged by the experimental period
203 (14 d period). The percentage of mean meal size above 750 g was estimated, the
204 criterion of 750 g was chosen based on the distribution of the meal size using all data
205 (all animals and all periods), 750 g was the average meal size. In addition, Nielsen
206 (1999) in their review observed a negative relationship between meal size and feeder
207 visits, and above 750 g of mean meal size this relationship is not linear, in consequence
208 above 750 g of meal size the number of visits to the feeder are reduced limiting total
209 daily feed intake. Concentrate efficiency data were transformed into log to achieve a
210 normal distribution. The means presented in the tables and figures correspond to non-
211 transformed data and, SEM and P-values correspond to the ANOVA analyses of the
212 transformed data. The percentage of each general activity was calculated, and the
213 average by day, pen, and scan obtained. Then, these data were transformed into natural
214 logarithms to achieve a normal distribution. The frequency of each social behavior was
215 calculated by summing by day, pen, and scan, and transformed into the root of the sum
216 of each activity plus 1 to achieve a normal distribution. The ANOVA analysis was

217 performed with transformed data, and the means shown in the tables correspond to the
218 back transformed data.

219 Performance, eating behavior, animal behavior and concentrate **intake** were analyzed
220 using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC). The model
221 included initial BW as a covariate, treatment, period (14-d period), and the interaction
222 between treatment and period and fattening cycle **(block),** as fixed effects, and the
223 interaction between **period** and pen and the 3-way interaction between **pen, period and**
224 **treatment** as random effects. Period was considered a repeated factor, and for each
225 analyzed variable, animal nested within the interaction between treatment and pen (the
226 error term) was subjected to **different** variance-covariance structures: compound
227 symmetry, **heterogeneous compound symmetry**, autoregressive order one,
228 **heterogeneous autoregressive**, and unstructured. **The diagonal elements of the UN**
229 **structure were examined to detect signs of heterogeneous variances across**
230 **time. Heterogeneity was not detected for any of the variables analyzed**. The
231 covariance structure that yielded the smallest Schwarz's Bayesian information criterion
232 was considered the most desirable analysis. **The covariate*trt has been checked and**
233 **the term was removed from the model when not significant. Hot carcass weight**
234 **was analyzed using a mixed-effects model (Version 9.2, SAS Inst., Inc., Cary, NC)**
235 **including initial BW as covariate, treatment and fattening cycle as fixed effects,**
236 **and pen as a random effect.**

237 **Analyses of categorical variables** (carcass classification, rumen health parameters,
238 hepatic abscesses, and percentage of meal size above 750 g) **an independent** Chi-
239 square-test was used.

240 Differences were declared significant at $P < 0.05$, and trends were discussed at $0.05 \leq P$
241 ≤ 0.10 for all models.

RESULTS

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).

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 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**).

No interactions between treatment and time were observed (**Table 2 and 3**) 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%).

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 6**). Carcass quality data are presented in **Table 6**. Dressing percentage (52.85 % ± 0.182), carcass conformation and fatness were not affected by treatment.

Animal Behavior

General Activities. General activities are showed in **Table 5**. 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.**

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.

289 ***Active Behavior.*** In the growing phase, during the visual scan observation period of 2 h,
290 no differences were observed for self-grooming and social behavior (14.27 ± 0.89
291 times/15 min and 5.16 ± 0.64 times/15 min, respectively) between treatments. Bulls of
292 the C group exhibited more ($P < 0.05$) oral non-nutritive behaviors (4.85 ± 0.78
293 times/15 min) **than BF bulls** (3.62 ± 0.78 times/15 min) (**Figure 1**). All behaviors
294 related to agonistic interactions were statistically different during this phase (**Figure 2**).
295 The frequency of fighting behaviors was **higher** ($P < 0.05$) in C bulls (5.25 ± 1.03
296 times/15 min) than in BF bulls (3.77 ± 1.03 times/15 min). Butting tended to be **higher**
297 ($P = 0.09$) for C group (3.01 ± 0.35 times/15 min) compared with BF group (2.21 ± 0.35
298 times/15 min), and an interaction ($P = 0.05$) between treatment and day was observed
299 for this behavior. Displacement interactions were lesser ($P < 0.05$) exhibited by C group
300 (0.18 ± 0.09 times/15 min) compared with BF group (0.27 ± 0.09 times/15 min).
301 Chasing and chasing-up interactions were **higher** ($P < 0.01$ and $P < 0.05$, respectively)
302 in the C bulls (0.48 ± 0.12 times/15 min and 0.11 ± 0.05 times/15 min, respectively)
303 than in the BF group (0.14 ± 0.12 times/15 min and 0.02 ± 0.05 times/15 min,
304 respectively), but these behaviors were occasionally exhibited. **No differences in**
305 **sexual behaviors (flehmen, attempt to mount, complete mounts) were observed in**
306 **this phase** (**Figure 3**).

307 During the finishing phase (from 113 d to 168 d), no differences were observed for self-
308 grooming behavior (7.39 ± 0.88 times/15 min) between treatments, whilst social and
309 oral behaviors were **higher** ($P < 0.01$ and $P < 0.001$, respectively) in bulls of the C
310 group (7.37 ± 0.76 times/15 min and 5.33 ± 0.54 times/15 min, respectively) compared
311 with BF bulls (4.81 ± 0.76 times/15 min and 2.52 ± 0.54 times/15 min, respectively)
312 (**Figure 1**). Regarding agonistic behavior (**Figure 2**), fighting and butting interactions
313 were **higher** ($P < 0.001$ and $P < 0.001$, respectively) in C group (8.50 ± 1.47 times/15

314 min and 6.29 ± 0.87 times/15 min, respectively) than in BF group. Although chasing
315 interactions occasionally occurred, bulls from the C group (0.64 ± 0.09 times/15 min)
316 exhibited **higher** ($P < 0.001$) interactions than BF bulls (0.04 ± 0.09 times/15 min).
317 Flehmen and complete mounts were **higher** ($P < 0.01$ and $P < 0.05$, respectively) in C
318 bulls (4.35 ± 0.76 times/15 min and 1.81 ± 0.29 times/15 min, respectively) than in BF
319 bulls (2.60 ± 0.76 times/15 min and 0.69 ± 0.29 times/15 min, respectively), whereas
320 attempt to mount interactions tended to be **higher** ($P = 0.10$) in bulls of the C group
321 (2.02 ± 0.57 times/15 min) compared with BF group (0.96 ± 0.57 times/15 min) (**Figure**
322 **3**).

323 ***Macroscopic Rumen Evaluation and Liver Abscesses***

324 At the slaughterhouse, color of rumen wall tended ($P = 0.06$) to be lighter for BF bulls
325 (1.27% classified as color "5") compared with C (9.76% classified as color "5").
326 Baldness areas presence in the rumen were lesser ($P = 0.01$) in BF group (48.1%) than
327 in C (67.1%) (**Table 7**). No differences were observed for liver abscesses between
328 treatments at the slaughterhouse (**Table 7**).

329 **DISCUSSION**

330 ***Intake, eating pattern and performance***

331 Bulls supplemented with flavonoids reduced concentrate intake throughout the study
332 compared with control group, and surprisingly, eating pattern parameters did not
333 differed between treatments. As concentrate intake is the consequence of the meal size
334 and daily number of visits to the feeder, these parameters were more deeply studied.
335 When meal sizes above 750 g were analyzed, no differences were observed in the
336 growing phase (from 0 d to 112 d) between treatments. Contrary, during finishing phase
337 (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 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.

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**).

Animal Behavior

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,

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

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

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

432 ***Social Behavior.***

433 Animal abnormal behaviors are indicative of poor welfare. In cattle, aggressive and oral
434 non-nutritive behaviors have been described as indicators of poor welfare (Gonyou et
435 al., 1994; Devant et al., 2016), frustration and discomfort. Microbiota, inflammation and

436 diet (Haagensen et al., 2014; Wiley et al., 2017), may affect behavior in humans and
437 other animals, and gut-brain-microbiota axis has been proposed as a communication
438 network between brain, digestive system and its microbiota. In this study, C bulls
439 exhibited more ($P < 0.05$) oral non-nutritive behaviors than BF animals. This behavior
440 of licking objects with non-nutritional finality has been described as an abnormal oral
441 behavior in cattle, and a gut dysfunction has been suggested as one of the possible
442 causes (Bergeron et al, 2006). Devant et al. (2016) reported that bulls fed high-
443 concentrate diet without access to straw increased oral behaviors, and this was related to
444 an increase in rumen lesions, low rumination activity and low pH. In agreement with
445 Devant et al. (2016), supplementation with flavonoids in previous studies has showed
446 an increase in straw consumption and rumen pH (Balcells et al., 2012), in the present
447 study in macroscopic rumen wall extraction indicated that wall was less damaged.
448 Moreover, the reduction of large meal sizes (less pH fluctuations) and the increased
449 time devoted to eat straw (reducing time devoted to perform other behaviors and **higher**
450 insalivation) in BF bulls during the finishing phase could explain a reduction of these
451 oral behaviors.

452 Bulls supplemented with flavonoids also exhibited less aggressive behaviors (agonistic
453 interactions), as fighting and butting, and less sexual interactions as well. Devant et al.
454 (2016) observed that diet presentation (pellet or meal) and straw provision (with or
455 without) in cattle fed high-concentrate diets modified the expression of different genes
456 (*ffar3*, *ppyr1, adra2c, occluding* and tnf_{α}), and suggested that the rumen could be
457 involved in the crosstalk between digestive system and brain modifying animal
458 aggressive and sexual behavior. The expression of the gene *ffar3* is stimulated by VFA,
459 mainly for propionic acid, and this gene stimulates the secretion of serotonin (Evans et
460 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.

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.

Conclusions

In conclusion, Bioflavex® CA 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

flavonoids spent more time eating straw. 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, flavonoids supplementation reduced agonistic behaviors throughout the study, and sexual interactions during the finishing phase.

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Table 1. Ingredients and nutrient composition of the concentrates.

Item	Growing	Finishing
Ingredients, %		
Corn grain meal	39.98	44.96
Gluten feed	23.00	21.31
Barley grain meal	13.82	10.87
Wheat	11.02	11.01
Beet pulp	4.90	4.99
Palm oil	2.38	2.75
Soybean meal	1.60	1.60
Calcium carbonate	1.60	1.29
Urea	0.80	0.42
Bicarbonate	0.40	0.40
Vitamin premix	0.30	0.20
Salt	0.20	0.20
Nutrients, dry matter (DM) basis		
CP, %	15.2	13.6
EE, %	5.3	5.8
Ash, %	6.1	5.5
NDF, %	18.5	17.8
TDN, %	88.6	89.3
PDIE, g/kg	97.1	97.7
PDIN, g/kg	101.4	102.1
NFC, %	54.8	57.2
UFC/kg	1.17	1.19

677 **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 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.
Sterertypies	Oral stereotypies	Tongue rolling, stereotyped licjing or bitting any equipment

678

679

680 **Table 3**. Performance, concentrate intake, and eating behavior of Holstein bulls fed
681 high-concentrate diets with or without BIOFLAVEX® CA supplementation from
682 4 to 9 mo of age.
683

	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

684 [1] Control = non-supplemented, BF = concentrate supplemented with BIOFLAVEX®
685 CA at 0.04%.
686 [2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by
687 time interaction effect.
688

689 **Table 4**. Performance, concentrate intake, and eating behavior of Holstein bulls fed
690 high-concentrate diets with or without BIOFLAVEX® CA supplementation from
691 9 to 11 mo of age.
692

	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 (168 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, g/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

693 [1] Control = non-supplemented, BF = concentrate supplemented with BIOFLAVEX®
694 CA at 0.04%.
695 [2] T = treatment effect; Time = time effect (period of 14 d); T x Time = treatment by
696 time interaction effect.
697

Table 5. Percentages of general activities (%) of Holstein bulls fed high-concentrate diets with or without BIOFLAVEX® CA 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 BIOFLAVEX® CA 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 6. Carcass quality of Holstein bulls fed high-concentrate diets with or without BIOFLAVEX® CA supplementation.

Item	Treatment[1]		SEM	*P-value*[2]
	Control	BF		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 BIOFLAVEX® CA at 0.04%.

[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.

738 **Table 7**. Macroscopically observations of the rumen of Holstein bulls fed high-
739 concentrate diets with or without BIOFLAVEX® CA 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		

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742 [1] Control = non-supplemented, BF= concentrate supplemented with BIOFLAVEX® CA
743 at 0.04%.
744 [2] T = treatment effect.
745 [3]Adapted from Gonzalez et al. (2001): Rumen color: 1= white; 5 = black.
746 [4]Adapted from Nocek et al. (1984).
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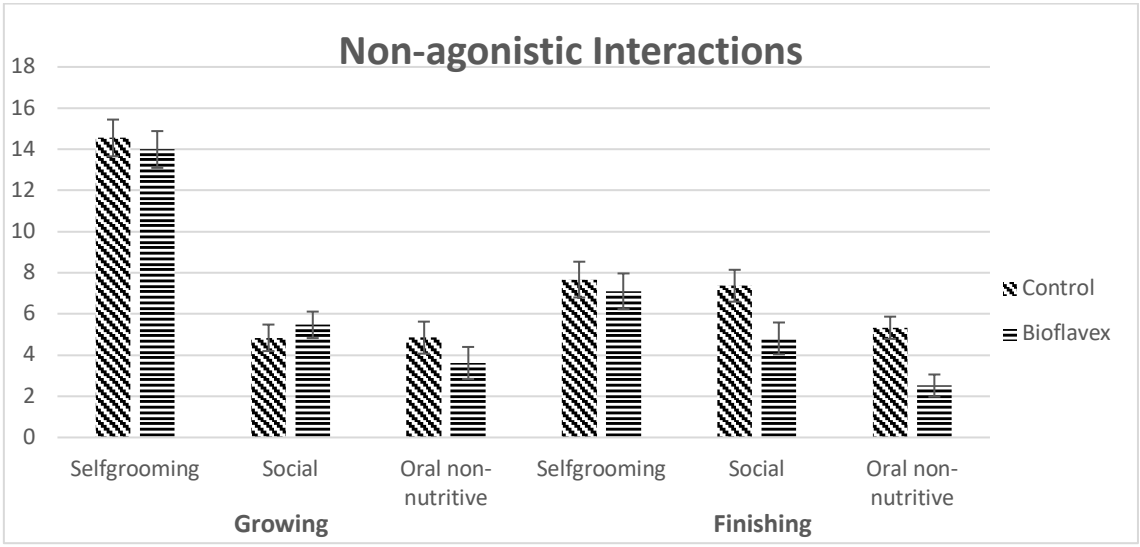
Figure 1. Non-agonistic interactions of Holstein bulls fed high-concentrate diets with or without BIOFLAVEX® CA supplementation.



753 **Figure 2.** Agonistic interactions of Holstein bulls fed high-concentrate diets with or
754 without BIOFLAVEX® CA supplementation.

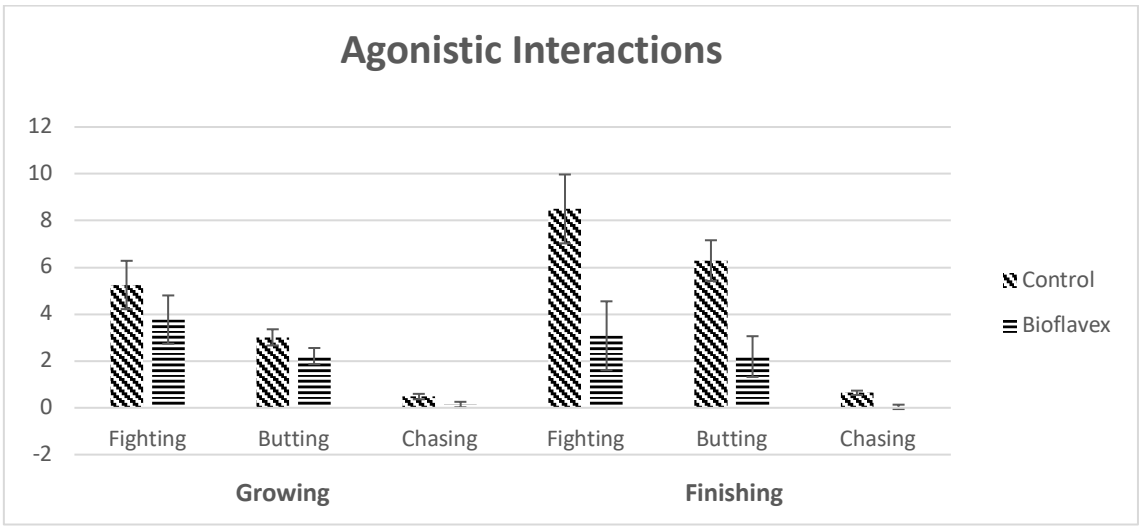


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Figure 3. Sexual interactions of Holstein bulls fed high-concentrate diets with or without BIOFLAVEX® CA supplementation.