Effects of partial substitution of barley with maize and sugar beet pulp on growth performance, rumen fermentation and microbial diversity shift of beef calves during transition from a milk and pasture regimen to a high-concentrate diet.

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Abstract

Effects of partial substitution of barley with maize and beet pulp (BP) on animal performance, rumen fermentation and rumen microbial populations were evaluated in thirty-nine newly weaned calves transitioned from a milk and pasture regime to a high-concentrate ration. Diets were: a barley-based growing diet (C); and two diets in which barley was partially replaced with maize and 10% BP (BP10) or 22% BP (BP22) on as fed basis. Wheat straw was offered *ad libitum* in all cases. Three adaptation protocols were followed: in Protocol 1 animals were abruptly shifted to the C diet, whereas calves in Protocols 2 and 3 were gradually adapted and received diets BP10 and BP22, respectively, for 10d; thereafter a 50:50 mixture of their diet and diet C until day 14, and finally, from day 15, diet C. All animals were slaughtered at a target live weight (LW) of *ca*. 500 kg. The experiment had then three phases (Ph): Ph1 (0-10d), Ph2 (11-14d) and Ph3 (15-slaughter). Concentrate intake and proportion of straw in the diet were affected ($P<0.01$) by the interaction dietary protocol by phase, showing animals in Protocol 3 the lowest concentrate intake and the highest straw proportion in Ph2. Daily gains, LW at the end of each phase, dressing percentage (59 ± 0.4%, on average) and total concentrate intake along the experiment (995 ± 21.3 kg DM, on average) were similar ($P>0.05$) among adaptation protocols. Daily rumen pH, and concentrations of lactate and volatile fatty acids were not affected by protocol. At the end of the trial, protozoa persisted in the rumen of all calves with an average concentration of 5.89 log cells/mL. Diversity dropped throughout the study; most protozoa belonged to genera *Entodinium* (97.5%) and *Isotricha* (1.7%), with minor presence of *Dasytricha* (0.05%), *Epidinium* (0.43%) and Subfamily *Diplodiniinae* (0.23%). Adaptation protocol had no effect on rumen bacterial population or diversity; however, both were affected by day of sampling indicating a shift in bacterial community in response to the concentrate inclusion. Although diversity dropped with concentrate inclusion, there was an increase after d10. In conclusion, the application of different protocols to adapt beef cattle to high-concentrate diets had no effect on animal performance and rumen fermentation which were not negatively affected by the abrupt transition to

the high-concentrate diet. This suggests that calves might cope with those feeding conditions probably due to the microbial adaptions to a new rumen environment.

Keywords: beef calves, barley replacing, transition, rumen fermentation, rumen microbiota.

1. Introduction

Conventional beef production in Spain is commonly carried out under intensive conditions. Beef calves are reared with their mothers on pasture and allowed to suckle freely for 5-7 months of age. After weaning, these calves face a feeding challenge since they are immediately shifted to a cereal-based high-concentrate diet. Under farm conditions, the transition period involves the first 14-21 days after the calves arrive in the feedlot where they receive a high-grain adaptation compound feed to stimulate the rumen papillae development and to acclimatise to the fattening concentrate (de Blas et al., 2008). Adaptation diets containing high proportions of starch are rapidly fermented resulting in high VFA and/or lactate concentrations, which contribute to increased risk of acute or sub-acute ruminal acidosis (SARA; Nagaraja and Titgemeyer, 2007). The transition is a challenging period for the rumen microbiota since it undergoes through an important dietary shift, which is known to be one of the most driving factors affecting rumen bacterial composition (Weimer, 2015). A gradual transition (Bevans et al., 2005), the use of feedstuffs with different rate of starch fermentation, and the substitution of cereals with high-energy by-products have been proposed as viable strategies to reduce the incidence of acidosis (González et al., 2012), and to improve the rumen health during this period, which affects animal health and production during the whole feeding cycle (Brown et al., 2006). The choice of the main cereal determines the acidogenic power of the diet (González et al., 2012). In this sense, maize grain has a lower rate and extent of ruminal degradation than barley grain (Herrera-Saldana et al., 1990). Previous studies have addressed the effect of replacing barley with maize as the prevailing cereal on rumen fermentation characteristics (Khorasani et al., 2001; Gimeno et al., 2015) but results are inconsistent. On the other hand, sugar beet pulp (BP) is a non-forage fibre source rich in neutral detergent fibre (NDF) and pectic substances (*ca.*25% of DM; FEDNA, 2010)

which chemical structure confers a high buffer capacity that can alleviate the over-acidification in the rumen. Pectin fermentation, although extensive and rapid, produces little or no lactate and gives an acetate/propionate ratio higher than starch (Van Soest et al., 1991). *In vitro* studies have reported that BP possesses a stabilizing effect of rumen pH and an acetogenic capacity (Marounek et al., 1985; Amanzougarene et al., 2017), which has been supported by *in vivo* studies with dairy cows (Mahjoubi et al., 2009) and beef steers (Mojtahedi and Danesh Mesgaran, 2011). Little is known about the effect of partial substitution of barley with maize and BP in adaptation diets to a high-concentrate regimen in beef cattle. This practice may be beneficial in raising and stabilizing pH during the transition period, which comprises a substantial risk of reaching low reticulo-ruminal pH for newly weaned calves that are not adapted to high-grain diets. On the other hand, although previous research has already explored the rumen microbiome dynamics during transition from forage to concentrate rations (Petri et al., 2013; Fernando et al., 2010), or during an acidotic challenge (Petri et al., 2013; Nagata et al., 2018), there is no much information on weaned calves transitioned from a milk/grass regime to a high-concentrate diet. We hypothesized that partially replacing barley with maize and BP in the adaptation diet of beef calves during transition from milk and pasture to a conventional high-concentrate ration would attenuate ruminal fermentation and reduce the risk of acidotic conditions during this critical period. Therefore, the objectives of the current experiment were to study the effect of the partial substitution of barley grain with maize and beet pulp in the adaptation diet of beef calves during transition from milk and pasture to a conventional high-concentrate ration on feed intake and daily gains, rumen fermentation, and rumen microbial composition of beef calves during transition from milk and pasture to a conventional high-concentrate ration.

2. Materials and Methods

2.1 Animals

Thirty-nine autumn-born Limousine crossbred male calves reared with their dams on pasture, and allowed to suckle freely while they were grazing, were used. Calves (250 ± 3.9 kg (SE)) were

abruptly weaned and transported to the Servicio de Experimentación Animal (University of Zaragoza) where they were individually housed in pens (1.7 x 3.4 m) provided with slatted concrete floor, automatic water dispenser and two separate troughs for concentrate and wheat straw. From the arrival to the facilities, calves were offered fescue (*Festuca pratensis*) hay (g/kg: organic matter, OM 904; crude protein, CP 128; neutral detergent fibre, NDF 654; acid detergent fibre, ADF 322; lignin 58) *ad libitum* for their adaptation to the farm environmental conditions. One week after their arrival, twelve calves were fitted in the dorsal sac of the rumen with a 150 mm long, 15 mm internal diameter (i.d.) permanent cannula, and allowed two more weeks for recovery from surgery. Then, all animals were blocked by body weight and randomly assigned to one of the three experimental diets described below (thirteen animals, including four cannulated, per experimental diet, ensuring homogeneous average weight and standard deviation per group). Animals began the experiment with a live weight (LW) of 258 kg (± 3.9 kg (SEM)), and an age of 215d (± 5.7 d (SEM)). Animal care, handling and surgical procedures were approved by the Ethics Committee of the University of Zaragoza. The care and management of animals were performed according to the Spanish Policy for Animal Protection RD 1201/05, which meets the EU Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

2.2 Diets and experimental procedures

Three isoenergetic and isoproteic diets were used: a control barley-based growing compound feed (C; including 9.3 g palm oil/kg and 57 g corn gluten feed/kg), and two concentrates formulated with an inclusion of either 10% (BP10; including 54 g corn gluten feed/kg, but no palm oil) or 22% (BP22; including 29.5 g palm oil/kg and 92 g corn gluten feed/kg) of dried sugar beet pulp on an as fed basis (Table 1). Diets were formulated taking into account the different starch degradation rates of the ingredients, and starch/NDF ratios. In this sense, 66% of barley was replaced in the BP10 diet for 49.5% maize and 16.5% BP. In the BP22 diet, there was a 75% barley replacement (30% with maize, 37% BP, and the rest with other ingredients: corn gluten feed, palm oil, etc.). Three

adaptation protocols were followed: in Protocol 1 animals were abruptly shifted from milk and grass to the C diet. Animals in Protocol 2 received diet BP10 and those in Protocol 3 diet BP22 for 10 days, then a mixture of 50:50 of their diet and diet C until day 14, and finally, from day 15, diet C. All animals were slaughtered at a target LW of *ca*. 500 kg. The experiment was therefore divided into three phases (Ph): Ph1 from days 0 to 10, Ph2 from day 11 to day 14, and Ph3 from day 15 until slaughter on day 154. Hence the transition period involved the first 14 d (Ph1 and Ph2).

Animals were weighed weekly prior to feed distribution. The average daily gain (ADG) was calculated as the regression coefficient of individual LW on time. Concentrates (ground to 3.5 mm) were offered *ad libitum* once a day at 09:00 h, and the amount was daily adjusted to ensure at least 10% of refusals. Wheat straw, chopped coarsely to approximately 10 cm in length, was offered three times daily (9:00 h, 12:00 h and 18:00 h) to ensure *ad libitum* access. The initial amount of concentrate offered per animal was 8 kg fresh matter. The amounts of concentrate and straw offered to each animal were recorded daily throughout the experiment. Straw intake was only recorded until the end of the transition period (end of Ph2). Representative samples of each compound feed and straw were taken weekly throughout the experimental period, pooled at the end of the experiment and analysed for chemical composition (Table 1). Concentrate and straw refusals (these latter only during transition) were collected daily and weekly, respectively, pooled on an animal basis and weighed weekly. Representative samples of offered feeds and refusals were dried at 104ºC for 24 h, to determine individual dry matter intake (DMI). Concentrate conversion ratio (CCR) was calculated from total amount of concentrate DMI and weight gained by the animals throughout the study. Aiming to collect rumen epithelium samples for a histological study, a subset of four non-cannulated animals from each treatment was randomly chosen and euthanized on d21 following the European Guidelines for Animal Welfare (Directive 86/609 EEC). No relevant histopathological findings were found and thus results will not be commented.

Feed intake pattern of both concentrate and straw was measured on days 9 (Ph1), 14 (Ph2) and 20 (Ph3) of the experiment. Recording intervals were 9:00-11:00, 11:00-13:00, 13:00-15:00, 15:00-17:00, 17:00-19:00, 19:00-21:00 and 21:00 to 09:00 h of the next day for concentrates, and 9:00-13:00, 13:00-17:00, 17:00-21:00 and 21:00 to 09:00 h of the next day for straw.

2.3 Rumen sampling

Rumen fluid samples were obtained at 0 h (before feeding) on d0, at 0, 3, 6 and 9 h after feeding at the end of Ph1 and Ph2 (days 10 and 14), and thereafter in Ph3 on days 21, 30 and 42. Since on d0 we only collected rumen fluid at 0h, two different statistical analyses were conducted: one with all 0h over all sampling days to study the effect of the concentrate inclusion, and another with all days and sampling intervals except d0 to study the fermentation pattern of the different diets. About 200 mL were removed from the rumen of each animal on each sampling time, using a customized vacuum device connected to a 0.6 cm i.d. semi-rigid tube with 2-mm pores. Representative samples were taken moving the tube in all directions inside the rumen while sampling. A 5 mL subsample was taken and immediately frozen in liquid nitrogen at -80ºC until molecular analyses of bacterial DNA was carried out. Another subsample of 2 mL (only taken at 0 and 6 h after feeding) was pipetted with a wide mouth tip (3 mm) into a tube containing 2 mL of 18.5% formaldehyde for quantification of the rumen protozoa. Then, rumen fluid was strained through a 1-mm pore size metal mesh sieve, and pH was immediately measured using a portable pH-meter (model Seven2GO, Mettler-Toledo AG, Schwerzenbach, Switzerland). Rumen fluid aliquots were taken, in duplicate, for ammonia, lactic acid and volatile fatty acids (VFA) analysis, and were analysed following the procedures described by Gimeno et al. (2015).

2.4 Chemical and microbiological analyses

Samples of the different feedstuffs were ground in a hammer mill fitted with a 1-mm sieve size, and analysed following the procedures of AOAC (2005) for dry matter (DM; ref 934.01), organic

158 matter (OM; ref. 942.05), crude protein (CP; ref. 976.05) and ether extract (EE; ref. 2003.05).
159 Concentration of NDF was analysed using an Ankom 200 Fiber Analyzer (Ankom Technology) as
160 described by Mertens (2002), using α-amilase and sodium sulphite, and results were expressed
161 exclusive of residual ashes. Acid detergent fibre (ADF) and acid detergent lignin were analysed as
162 described by AOAC (2005; ref. 973.18), and Robertson and Van Soest (1981), respectively. Total
163 starch content was determined enzymatically from samples ground to 0.5 mm using a commercial kit
164 (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). Metabolizable energy (ME) of
165 wheat straw was calculated from ADF content according to the equation proposed by Mertens (1983)
166 (NE = 2.469-0.0351*%ADF; R^2=0.849; ME = NE/0.61), whereas ME of concentrates was calculated
167 taking into account the ingredient composition and ME values from FEDNA (2010). Only energy
168 intake of the transition period (Ph1 and Ph2; 0-14d) was considered as in Ph3 straw intake was not
169 recorded.

170 Rumen samples taken at 6h after feeding on days 10, 14, 30 and 42 of the experiment were
171 chosen for protozoal quantification. Optical observation and genera and species identification were
172 carried out as outlined by Dehority (1993). During each counting, the numbers of different genera in
173 the protozoal population were recorded, identified and grouped into the genera *Isotricha* and
174 *Dasytricha* from the family *Isotrichidae*; genus *Epidinium* from the Subfamily *Ophryoscolecinae*;
175 genus *Entodinium* from the Subfamily *Entodiniinae*; and genera *Diplodinium*, *Metadinium*,
176 *Eudiplodinium*, *Enoploplastron*, *Ostracodinium* and *Polyplastron* from the Subfamily *Diplodiniinae*.
177 Total protozoal and groups concentration for each calf were calculated and transformed into
178 logarithmic basis (log_{10}/mL) to assume normality before being subjected to statistical analysis;
179 however, for a better interpretation, relative abundances of different protozoal groups are presented
180 as percentage with standard error in the results section. Absence of total protozoa or any protozoal
181 group was considered as zero in the statistical analyses.

182

183 *2.6 DNA extraction and Ion Torrent sequencing*

184 Samples taken before feeding on days 0, 10, 14, 21 and 30 were chosen for sequencing
185 analyses using Ion Torrent Next Generation Sequencing (NGS). Samples were freeze-dried,
186 thoroughly mixed and disrupted (Mini-Bead Beater, Biospec Products, Bartlesville, OK, USA). The
187 microbial DNA was extracted using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen Ltd., West
188 Sussex, UK) following the manufacturer recommendations, except that samples were initially heated
189 at 95ºC for 5 min to maximize bacterial cell lysis. Concentration and purity of extracted DNA was
190 tested in Nanodrop ND-1000 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). Sequencing
191 of the 16S rRNA gene was conducted following the procedure described by de la Fuente et al. (2014)
192 and Schauf et al. (2018). Briefly, the bacterial V1-V3 hypervariable region was amplified by PCR
193 using barcoded fusion primer pairs 27F and 338R (Wang et al., 2014). Sequencing of the PCR
194 products was performed in the Ion Torrent Personal Genome Machine (PGM) system (Life
195 Technologies, Carlsbad, CA, US) using the Ion PGM Sequencing 200 kit v2 (Life Technologies,
196 Carlsbad, CA). Following sequencing, data were combined and sample identification numbers
197 assigned to multiplexed reads using Ion Reporter™ 5.10. Software (Thermo Fisher Scientific).
198 Sequencing amplicon reads were subjected to trimming, denoising, and chimera removal and
199 clustered into Operational Taxonomic Units (OTU) at 97% identity using UPARSE pipeline (Edgar,
200 2013). Reads were subjected to quality filtering (quality score of 20 in a 1 to 40 scale) and trimmed
201 at a maximum length of 250 bp. Taxonomic assignation of 16S rRNA sequences was established by
202 comparison against the Ribosomal Data Project II database (Cole et al., 2003), considering a bootstrap
203 value of 0.80 for annotation, leaving successive taxon levels as unclassified.
204 To maximize the comparability across samples, the number of reads was manually normalized to the
205 sample with the lowest number of reads (85,442). Numbers of reads of each microbial taxon were
206 $\log_{10}$-transformed (nº of reads + 1) prior to statistical analysis to assume normality; however, for a
207 better interpretation, relative abundances of different bacterial taxa are presented as percentage with

208 standard error in the results section. Two measures of diversity were calculated: Shannon index of
209 general diversity and richness according to the number of observed genera using R software v.3.5.3.

210 *2.7 Statistical analyses*

211 Data of final LW of each phase, concentrate DMI (CDMI), total CDMI, proportion of wheat
212 straw in the ration, ADG, CCR and dressing percentage were corrected by covariance using the initial
213 body weight and age as covariates. Concentrate intake, proportion of straw in the ration, ME intake,
214 ADG gain and LW at the end of each phase were analysed as repeated measures using the PROC
215 MIXED of SAS (SAS Inst. Inc., Cary NC, US, v 9.4) with protocol, phase and their interactions as
216 fixed effects, and animal as random. For total CDMI, CCR and dressing percentage protocol was the
217 fixed effect, and animal the random effect. Pattern of intake of concentrate and straw, rumen
218 fermentation variables, rumen protozoa abundance, bacterial taxa abundance (excluding those with
219 relative abundances lower than 0.1% of total sequences), different ratios between phyla, and diversity
220 indexes were analysed as repeated measures with the MIXED procedure with protocol, sampling time
221 interval nested within day (when applicable), sampling day and all possible interactions as fixed
222 effects, and animal as random. Sampling time nested within day or sampling day was used as repeated
223 measure, as appropriate. For bacterial taxa abundance, treatment means were compared under a
224 conservative Bonferroni-adjustment. The variance-covariance structure was selected based on the
225 lowest Akaike information criterion. For sequencing data, multivariate statistical analyses were
226 performed using package "vegan" from the R statistical program. To determine the impact of the diet
227 and time on overall microbial community structure, a non-parametric permutational multivariate
228 analysis of variance (PERMANOVA) was conducted based on the Bray-Curtis dissimilarity.
229 Spearman correlation coefficients were calculated to assess the relationships between the ruminal
230 fermentation characteristics at hour 0 and the log-transformed data of the main bacterial taxa and
231 protozoal concentrations using R software. Only Spearman correlations with $r \geq 0.50$ or $r \leq -0.5$ and

232 $P<0.05$ are shown. For all data, differences were considered significant if $P<0.05$, whereas
233 differences were considered to indicate a trend to significance when $0.05 \leq P \leq 0.10$.

234 **3. Results**

235 *3.1 Feed intake and animal performance*

236 No differences in concentrate intake between treatments were found in Ph1 and Ph3, but in
237 Ph2 animals on Protocol 3 showed the lowest values. As a result, the interaction between dietary
238 protocol and phase was significant ($P<0.01$; Table 2). Proportion of straw on total DMI was also
239 affected by the interaction between adaptation protocol and phase ($P<0.01$; Table 2), showing calves
240 in Protocol 3 the highest figures in Ph2. Hence the amount of ME ingested (from concentrate and
241 straw) during transition (0-14d) was lower for animals in Protocol 3 during Ph2 (62, 67 and 52 MJ of
242 ME for Protocols 1, 2 and 3, respectively), with no differences between protocols for Ph1 and Ph3
243 (significant interaction protocol x phase; $P<0.01$). The pattern of intake of concentrate (Figure 1) was
244 not affected by adaptation protocol ($P=0.64$) or sampling day ($P=0.55$), but it was affected by time
245 interval ($P<0.001$) and animals consumed 31% of the concentrate during the first 4 h. The pattern of
246 intake of wheat straw (Figure 1) was affected neither by dietary protocol ($P=0.74$) nor by sampling
247 day ($P=0.61$), but was also affected by time interval ($P<0.001$). To this respect, the highest intake of
248 straw was observed during the interval of 12-24 h after feeding, when calves consumed, on average,
249 34% of the daily straw intake. Overall, the highest total DM intake was observed during the first 4h
250 after the morning feeding ($P<0.001$) when animals consumed, on average, 30% of the ration,
251 decreasing to 19% in the following 4h. Total CDMI at the end of the study did not differ among
252 groups (1002, 978 and 1010 kg of DM for Protocols 1, 2 and 3, $P=0.69$). Regarding daily gains, no
253 differences were found among protocols ($P=0.64$; Table 2), and ADG increased over time (0.65, 0.73
254 and 1.58 kg/d in Ph1, Ph2 and Ph3, respectively, $P<0.001$). All groups exhibited similar ($P> 0.10$)
255 LW at the end of each phase (Table 2), CCR (4.21 ± 0.100) and dressing percentage (average 59 ±
256 0.4%).

3.2 Rumen fermentation

Analyses of hour 0 of all sampling days showed that concentrate inclusion, regardless the diet, modified the rumen environment (data not shown). Compared to d0, rumen pH decreased ($P<0.001$), lactate ($P<0.001$) and total VFA ($P<0.01$) increased, and acetate ($P<0.001$) and the ratio acetate/propionate ($P<0.05$) decreased on the following days. Shifts towards increased propionate ($P<0.05$), butyrate ($P<0.001$), valerate ($P<0.001$), and branched-chain fatty acids (BCFA: sum of isovalerate and isobutyrate; $P<0.01$) were also found. Ammonia concentrations at hour 0 remained stable throughout the study ($P=0.90$). All rumen fermentation variables (except for ammonia (P=0.06) and lactate (P=0.74) concentration, and molar proportions of propionate (P=0.08) and valerate (P= 0.30)) were affected ($P<0.05$) by sampling time after feeding (data not shown), whereas adaptation protocol or its interaction with sampling time was not significant in any case (P>0.10). Rumen pH decreased (P<0.001) to a major extent during the first 3 h, on average -0.25 units. Sampling day affected ($P<0.05$) lactate concentration (highest at the end of transition), VFA concentration (which decreased over time) and proportions of propionate, valerate and BCFA, and rumen pH tended to differ across days (P=0.08). Given that the lowest pH was always registered at 9 h after feeding, coinciding with the highest VFA concentration, rumen fermentation variables at hour 9 after feeding were analysed separately for an easier comparison of the fermentability of the diets. Rumen pH at 9 h did not differ among protocols or sampling days (Table 3), whereas lactate concentration was not affected by protocol but it was by sampling day, the lowest value appearing on day 35. Ammonia concentration at 9 h was highly variable among animals and tended to be affected by the interaction between protocol and sampling day ($P=0.052$; Table 3). Total VFA and proportions of the main VFA were not affected by the adaptation protocol but differed across sampling days (except for butyrate; Table 4).

282 *3.3 Rumen microbial population*

283 Rumen protozoa concentration was not affected by protocol (P=0.76) or sampling day
284 (P=0.19), and averaged 6.22, 6.00, 5.21 and 5.89 log cells/mL on days 10, 14, 35 and 42 of the
285 experiment. Protozoal population was highly variable among animals and days of sampling, and
286 ranged from 2.73 to 6.83 log cells/mL. Only one calf from group BP22 was defaunated on d35, but
287 recovered a stable population thereafter. It is worth mentioning that at the end of transition (d14) all
288 animals harboured a consistent protozoal population and diversity despite the abrupt dietary change.
289 On d42, the observed protozoa belonged mostly to the genera *Entodinium* (97.5 ± 0.72%); although
290 *Isotricha* was also present (1.74 ± 0.47%). Protozoa of the genera *Dasytricha* (0.05 ± 0.057%),
291 *Epidinium* (0.43 ± 0.372%) and those of the Subfamily *Diplodiniinae* (0.23 ± 0.135%) were virtually
292 absent on d42. No significant correlations were found with protozoal concentrations and rumen
293 fermentation variables.

294 Regarding bacterial population, a high individual variability was found among calves (CV
295 from 29% to 270%). Collectively, four phyla represented more than 95% of the total sequences:
296 *Bacteroidetes* (45.8% ± 3.88%), *Firmicutes* (43.0% ± 3.11), *Actinobacteria* (5.9% ± 3.10), and
297 *Proteobacteria* (3.5% ± 1.66). Of the two major phyla, *Bacteroidetes* were dominated by a single
298 genus (*Prevotella*), while *Firmicutes* comprised several families and genera (Table 5). The
299 PERMANOVA revealed no effect of adaptation protocol (P=0.46, R^2=0.03) but a significant effect
300 of time on overall microbial composition (P<0.001; R^2=0.09). The effect of protocol was not
301 significant for any taxon; however, the abundance of most taxonomic levels differed across sampling
302 days (Table 5). Of the four major phyla, only *Firmicutes* varied over time (P<0.001), and showed
303 lower abundance on d30 compared to d0, and the similar trend was observed on minor phyla such as
304 *Chloroflexi* (P<0.001), *Synergistetes* (P<0.001), and *Tenericutes* (P<0.001). *Proteobacteria* and the
305 ratio *Proteobacteria* (calculated as the abundance of *Proteobacteria* sequences divided by the sum of
306 the abundance of *Firmicutes* and *Bacteroidetes* sequences) remained stable over time (P>0.05), and

two C calves showed ratios higher than 0.19 on d14. The ratio *Firmicutes*/*Bacteroidetes* tended to differ over time (P=0.08), and on d30 it was lower compared to day 0 (1.63 *vs.* 0.89; Table 1 of Supplementary material). After concentrate inclusion, on d10 some genera appeared in the rumen: *Bifidobacterium*, *Lactobacillus*, *Roseburia*, *Sharpea* and *Succinomonas*. During the transition period (d0 to d14), regardless the adaptation protocol, there was a decline ($P<0.05$) in the members of the phyla *Chloroflexi*, *Synergistetes* and *Tenericutes*, and of the genera *Atopobium*, *Clostridium*, *Butyrivibrio*, *Coprococcus*, *Pseubutyrivibrio* and *Streptococcus*. In addition, there was an increase of *Bifidobacterium*, *Prevotella*, *Roseburia*, *Lactobacillus*, *Selenomonas*, *Anaerovibrio* and *Sharpea* on d14 compared to d0 ($P<0.05$). Overall, on d30 compared to d0, at the genus level, *Bifidobacterium*, *Roseburia*, *Lactobacillus* and *Succiniclasticum* increased ($P<0.001$); and *Eubacterium*, *Butyrivibrio*, *Pseudobutyrivibrio*, *Coprococcus*, *Selenomonas* and *Anaerovibrio* decreased ($P<0.001$; Table 1 of Supplementary material). Diversity indexes (Shannon and genera richness) were not affected by adaptation protocol ($P>0.05$), but were altered across sampling days ($P<0.05$; Table 5). Correlation analysis between relative abundances of the bacterial taxa and rumen fermentation variables were not consistent across protocols, and only the abundance of few taxa exhibited a consistent correlation under the three groups (Table 6). For instance, family *Bifidobacteriaceae* positively correlated with butyrate and valerate, and genera *Fibrobacter* and *Butyrivibrio* positively with rumen pH and negatively with valerate.

Certain bacterial genera were detected ubiquitously in the rumen of all calves across the experiment, and therefore, were defined as the 'core microbiome'. The abundance of the shared taxa in the overall bacterial community was highly diverse ranging from 0.01% to 55% of total bacteria. Treatments did not show differences regarding core taxa.

4. Discussion

In this study, we have evaluated three protocols to adapt beef calves from a milk and forage diet to a high-concentrate ration. To do that, barley was partially replaced by maize and beet pulp in

the adaptation diet during the transition period. Animals in Protocol 1 were considered to be abruptly shifted to the fattening diet, whereas those in Protocols 2 and 3 were supposed to be gradually transitioned. Adaptation diets with lower acidogenic capacity due to a slower degradation rate of starch (González et al., 2012), and to a ratio starch to NDF lower than 2:1 (Bacha, 2002), are desirable to prevent ruminal acidosis. Diet C had the greatest acidogenic power since barley possesses a higher rate of fermentation than maize (Herrera-Saldana et al., 1990), and this diet had a ratio starch:NDF of 3:1. Although diet BP10 showed the same starch/NDF ratio, its acidogenic capacity was lower because of the lower proportion of barley and the inclusion of BP itself. On the other hand, beet pulp is unique in its composition and possesses a high buffer capacity, which is partly due to the galacturonic acid in its structure and its high fibre content (Jasaitis et al., 1987; Van Soest et al., 1991). Attention must be drawn to the fact that diets C and BP22 also included palm oil (9.3 and 29.5 g/kg, respectively) and this might have helped to alleviate the risk of ruminal acidosis in animals consuming those diets. Also, corn gluten feed was included in the formulae (57, 54 and 92 g/kg for diets C, BP10 and BP22, respectively), and the higher level in diet BP22 could have also had an effect on rumen fermentation.

4.1 Feed intake and animal performance

Concentrate intake during Ph1 and Ph3 were similar among protocols. However, in Ph2 (when calves in Protocols 2 and 3 received a 50:50 mixture of their BP diets with the C diet) calves in Protocol 3 showed the lowest intake of concentrate and consumed more straw, suggesting an autoregulation mechanism to increase the buffering capacity of the rumen (González et al., 2012) in response to the acidogenic capacity of the new diet. In Ph3, animals in Protocol 2 seemed to be more adapted to ration C, which might be due to the higher acidogenic power of the previous BP10 diet. Throughout Ph3, DMI and daily gains were similar among protocols. Similarly, Bevans et al. (2005) reported that a gradual or rapid adaptation to high-concentrate diets had no effect on DMI thereafter in the feedlot period.

With respect to the use of barley or maize in the diet, previous studies have also found no differences on intake among groups in steers (Surber and Bownman, 1998; Gimeno et al., 2015) or lambs (Yahaghi et al., 2012) fed corn- or barley-based diets. Regarding barley replacement with BP, Bodas et al. (2007) reported lower intake in fattening lambs when replacing 12% of barley with BP. In contrast, Maktabi et al. (2016) replaced BP for grains (barley and corn) in the starter diet of dairy calves and reported higher DMI.

Regarding the pattern of intake of concentrates and wheat straw, no differences were found among protocols or phases in agreement with the results reported by Gimeno et al. (2015), who studied the effect of the main cereal (proportions of barley to maize 75:25 or 25:75) on the rate of intake of concentrate and straw in beef steers. These authors attributed the lack of differences to the higher straw intake observed in animals receiving the barley-based concentrate. In the present experiment, animals consumed substantially more straw (*ca.*20%) than that reported in other experiments with animals fed high-concentrate diets (*ca.*5% in Devant et al., 2000; *ca.*7% in Gimeno et al., 2015) and this could explain the lack of differences between adaptation protocols.

4.2 Rumen fermentation variables

Results obtained from the analyses of rumen fermentation variables at hour 0 throughout the experiment mirrored the inclusion of concentrates, and were consistent with the general features reported after switching hay-adapted animals to a high-grain diet (Goad et al., 1998). Despite the different characteristics of the ingredients of the diets, no differences between adaptation protocols were observed for any of the daily average rumen fermentation characteristics or for their post-prandial evolution.

In agreement with our data, most studies did not detect differences on daily average rumen pH comparing barley- or corn- based diets in dairy cows (Casper et al., 1999; Khorasani et al., 2001) or beef cattle (Rotger et al., 2006; Gimeno et al., 2015). However, the extent of post-prandial decrease of rumen pH was higher when barley was the prevailing cereal (Khorasani et al., 2001; Gimeno et al.,

382 2015). As mentioned above, in the present study calves consumed a considerable amount of straw
383 that could explain the lack of differences in the extent of decrease of rumen pH after feeding, given
384 the different nature of diet ingredients. Regarding the BP inclusion, previous experiments reported
385 higher ruminal pH when BP increased at the expense of barley in dairy cows (Mahjoubi et al., 2009),
386 steers fed low-forage diets (Mojtahedi and Danesh Mesgaran, 2011), and fattening lambs (Bodas et
387 al., 2007). In Mahjoubi et al. (2009) and Mojtahedi and Danesh Mesgaran (2011) barley was the only
388 grain source accounting for 23% and 33% of DM, respectively, and thus replacing grain for BP in
389 these diets (from 33% to 100% replacement) is likely to result in an increase in pH due to the buffer
390 capacity of BP. The lack of effect of BP in our study is masked by the partial substitution of barley
391 with maize as well. In the study of Bodas et al. (2007) the higher pH could have probably been due
392 to the lower intake (P=0.03) promoted by the BP replacing 12% of barley and the numerically higher
393 straw intake in the BP group (14 vs. 19 g DM/d).

394 Lactate concentration in our study was similar among diets in agreement with the results of
395 Gimeno et al. (2015). In general, an overall increase of lactate concentration, which was not
396 accompanied by a low rumen pH (6.20 on average), was observed at the end of transition. Lactate
397 concentrations in our study (except for one animal that reached 7mmol/l in one sampling) were below
398 the risk values considered as benchmark of SARA (>5 mmol/L; Nagaraja and Titgemeyer, 2007).

399 Ammonia concentration was highly variable among animals but the main cereal or BP
400 inclusion in the diet did not exert any effect. Casper et al. (1999) reported lower ammonia
401 concentrations with barley-based diets which was attributed to the higher degradability of barley and
402 thus high available energy for microbes to capture N for protein synthesis. Other authors, however,
403 did not find any difference between barley and corn (Gimeno et al., 2015). In other studies, the
404 substitution of barley with BP was found to result in lower ammonia, which was attributed to the
405 more extensive utilization of ammonia by fibrolytic bacteria when diet contained BP (Mojthaedi and

406 Danesh Mesgaran, 2011). In contrast, others authors found similar ammonia concentrations in lambs
407 fed concentrates including or not BP (Bodas et al., 2007).

408 Overall, VFA concentration decreased over time despite the higher concentrate intake,
409 suggesting an increased absorption due to the adaptation of the rumen epithelium. Further, no effect
410 of adaptation protocol was found on VFA concentration or on proportions of individual VFA despite
411 differences in ingredient composition of the three diets. In agreement with our results, Rotger et al.
412 (2006) and Gimeno et al. (2015) did not report differences between barley and corn diets in beef
413 cattle; however, Surber and Bownman (1998) found higher VFA production (and higher butyrate and
414 lower propionate) with barley than with corn with identical DM intake between diets. In dairy cows,
415 Casper et al. (1999) reported greater VFA concentration in corn-fed cows, while Khorasani et al.
416 (2001) reported higher concentrations with barley, which was attributed to its higher degradability.
417 In our experiment, no effect of diet was found in proportions of individual VFA in agreement with
418 other reports (Rotger et al., 2006). The inconsistency among experiments might be explained by
419 differences in varieties of these cereals, the processing method, and the forage to concentrate ratio. In
420 previous studies, partially replacing barley with BP resulted in lower VFA concentrations (Bodas et
421 al., 2007; Mojtahedi and Danesh Mesgaran, 2011), and in some differences in the molar proportions
422 of VFA. Beet pulp is known by its acetogenic capacity (Marounek et al., 1985) and thus most studies
423 reported increased acetate/ propionate ratio (Mahjoubi et al., 2009; Maktabi et al., 2016). Others have
424 also reported higher butyrate, and authors speculated about the capacity of BP to stimulate butyrate-
425 producing microbiota (Mahjoubi et al., 2009; Mojtahedi and Danesh Mesgaran, 2011).

426 *4.3 Rumen microbial populations*

427 Regardless the applied dietary protocol, at the end of transition, a well-established protozoa
428 population persisted in the rumen of all animals despite the abrupt dietary change from milk and grass
429 to concentrate. These findings support those of Towne et al. (1990) and Hristov et al. (2001) who
430 observed that, although diversity decreased, a consistent protozoa population was present in steers

fed high-concentrate Further, our findings are consistent with those reported by Towne et al. (1990) who observed that how abruptly cattle are switched from an all-roughage to a finishing diet does not affect total protozoal concentration.

In agreement with other reports of rumen bacterial composition in beef cattle, we observed *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* to be the dominant phyla (Fernando et al., 2010; Petri et al., 2013). In our study, the *Firmicutes*/*Bacteroidetes* ratio fluctuated over time, and declined on d30 compared to d0, which is consistent with results reported by Fernando et al. (2010) since *Bacteroidetes* are usually more abundant with high-concentrate diets. A *Proteobacteria* ratio above 0.19 suggests an unstable microbial community (Auffret et al., 2017), and was detected on d14 in two calves in Protocol 1 in which *Proteobacteria* increased to as much as 42% and 19% of the total population, respectively. Unbalanced microbial populations can be associated with acidotic events. Probably, that might be the reason of those transient *Proteobacteria* abundances, since one of these calves showed a relatively high VFA concentration (155 mmol/L); and the other showed a relatively high lactate concentration (7.05 mmol/L) before the morning feeding. In addition, another BP10 animal had a relative abundance of *Actinobacteria* as high as 64%, a rumen pH of 5.79 and a high VFA concentration (257 mmol/L) at 0h on d30, likely indicating a transient acidotic episode. Other authors have found *Proteobacteria* and *Actinobacteria* to increase after an acidotic challenge (Petri et al., 2013). It is acknowledged that the rumen as an ecosystem responds to dietary changes with an ecological succession of compositional changes that develop more tolerant bacteria under the new environment (Weimer, 2015). In the present study, changes in bacterial composition as well as in the rumen fermentation variables paralleled the higher supply of fermentable substrates (mainly starch) favouring the growth of amylolytic and acid-tolerant bacteria (Goad et al., 1998). For instance, the introduction of concentrates promoted the appearance of some bacteria, such as *Bifidobacterium*, *Lactobacillus*, *Roseburia* and *Sharpea* on d10. Interestingly, these genera (and others that also increased during transition, such as *Prevotella*, *Selenomonas*, and *Anaerovibrio*) were positively

456 correlated with butyrate, propionate, VFA, and lactate, and negatively with rumen pH. Conversely,
457 genera *Butyrivibrio*, *Coprococcus,* and *Pseubutyrivibrio,* and phyla *Cloroflexi*, *Synergistetes,* and
458 *Tenericutes* decreased during the transition, and were positively correlated with rumen pH, and
459 negatively with VFA and lactate.

460 Genera richness and Shannon index deeply decreased after 10 days of concentrate inclusion
461 as recorded by other authors during the transition from forage to high-concentrate diet (Petri et al.,
462 2013; Nagata et al., 2018); however, the Shannon index slightly increased from d14 until the end of
463 the study. Along with the recovery of the abundances of some genera to initial values, the increase of
464 the Shannon index may suggest an adaptation and development of some genera that are tolerant to
465 the new environment. This is in agreement with the results reported by Nagata et al. (2018), who
466 studied the effect of four consecutive SARA challenges on rumen microbiota and diversity in Holstein
467 bulls, and reported that on the fourth challenge diversity indexes were greater than in the first. This
468 might refer to the resilient capacity of the rumen microbiota (Weimer, 2015). Together with the lack
469 of effect of diet on bacterial population or on rumen fermentation, the inconsistent correlations
470 between rumen fermentation and bacterial taxa across diets might suggest that bacteria can shift their
471 metabolic pathways depending on the substrates available without altering the final products (Taxis
472 et al., 2015). This feature refers to the principle of the functional redundancy of the rumen bacteria
473 (Taxis et al., 2015; Weimer, 2015).

474 Most taxa reported in our 'core microbiome' agreed with those previously reported by other
475 authors (Petri et al., 2013). Although the bacterial taxa that composed the rumen core were not equally
476 abundant and some genera in the core represented as low as 0.01% of total bacterial community, the
477 fact that were found in every animal across sampling days suggests that are important for rumen
478 function as already pointed out by Jami and Mizrahi (2012).

479 Overall, the most interesting finding obtained in the current experiment was the lack of effect
480 of the different adaptation protocols applied. All groups responded similarly to the transition without

impaired effects in terms of animal performance during the whole trial, ruminal fermentation, and rumen microbiota. Further, given the lack of differences in intake and daily gains during the transition, and the price of the concentrates (268, 271 and 292 €/ton for C, BP10 and BP22), it does not seem necessary, under practical farm management, to provide an adaptation concentrate and follow an adaptation protocol since no subsequent improvements or economic profit are achieved.

5. Conclusions

Collectively, our observations demonstrate that an abrupt transition from milk and grass regimen to a high-concentrate diet did not impair animal performance or rumen health of beef calves. Animals can compensate for the higher acidity of the diet increasing the straw intake, and it seems that the resilience of the rumen microbiota and the stability of the core microbes are key points to prevent the detrimental effects on rumen fermentation, and thus on animal performance. The use of barley or maize as the prevailing cereal or the inclusion of BP have no effect on rumen fermentation or microbial populations of beef calves, and their use will depend on market price or availability.

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Conflict of interest

We declare that we have no conflict of interest related to this manuscript.

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636 **Table 1.** Ingredient and nutrient composition of the experimental diets.

	Diets			
Ingredients (as fed basis), g/kg	C	BP10	BP22	Wheat straw
Barley	590.0	200.0	150.0	
Maize	150.5	466.1	333.4	
Beet Pulp		102.3	220.0	
Corn gluten feed (200gCP/ kg fresh matter)	57.0	53.9	91.9	
Soy meal (470 g CP/ kg fresh matter)	172.1	163.7	161.8	
Palm oil	9.3	-	29.5	
Urea	0.6	-	-	
Calcium carbonate	8.5	8.2	2.0	
Dicalcium phospate	5.0	-	6.4	
NaCl	5.0	3.8	3.0	
Vitamin-mineral premix[1]	2.0	2.0	2.0	
Nutrient composition (g/kg DM)				
OM	947.6	951.8	948.4	956.1
CP	171.2	174	170.9	18.2
EE	29.7	25.3	53.5	11
Starch	449	457	298	5
NDF	150.6	148.8	192.9	749
ADF	48.7	61.0	89.0	437
ME (MJ/kg DM)	11.41	11.45	11.75	6.40
Lignin (sa)	5.2	6.8	8.0	50.3

637 [1] Vitamin-mineral premix declared composition (per kg): $4x10^6$ IU vitamin A, $0.8x10^6$ IU vitamin
638 D3, $5x10^3$ IU alpha-tocopherol, 100 g sepiolite, 150 mg etoxiquin, 10 g Mn oxide, 7.5 g Fe carbonate,
639 20 g Zn oxide, 250 mg K-iodate, 300 mg Co, 2.5 g Cu, and 500 mg Na selenite, 10 mg antioxidants.
640
641 OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid
642 detergent fibre; ME, metabolizable energy; Lignin (sa), acid detergent lignin.

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Table 2. Effect of the adaptation protocol of beef calves to a high-concentrate diet[1] on concentrate and wheat straw dry matter (DM) intake (DMI), average daily gains (ADG) and live weight during transition and the fattening period.

		Phase 1	Phase 2	Phase 3		P-value		
		(0-10 d)	(11-14 d)	(15d-End)	SEM	Protocol	Phase	Protocol*Phase
Concentrate intake (kg DM/d)	Protocol 1	4.72	4.91a	6.75	0.2619	0.53	<0.001	<0.01
	Protocol 2	4.92	5.39a	6.57	0.3862			
	Protocol 3	4.72	3.95b	7.01	0.2317			
Straw intake[2] (% of total DMI/d)	Protocol 1	24	15b	-	0.3071	0.15	<0.001	<0.001
	Protocol 2	24	14b	-	0.3071			
	Protocol 3	22	24a	-	-			
ADG (kg/d)	Protocol 1	0.63	0.75	1.78	0.1003	0.64	<0.001	0.36
	Protocol 2	0.66	0.74	1.43	0.1003			
	Protocol 3	0.66	0.69	1.49	0.1015			
Live Weight at the end of each Phase (kg)	Protocol 1	268	279	507	5.8143	0.29	<0.001	0.70
	Protocol 2	270	278	499	5.8559			
	Protocol 3	268	274	488	5.8141			

[1] In Phase 1, calves received C (Protocol 1), BP10 (Protocol 2) or BP22 (Protocol 3) diets (see Table 1) from days 0 to 10; in Phase 2, animals on BP10 or BP22 groups received a mixture of 50:50 (w/w) of C and either BP10 or BP22 from day 11 to 14; Phase 3 comprised from day 15 to the end of the experiment (when animals reached *ca.* 500 kg), and all calves received diet C.

[2]Straw intake was recorded only during Phases 1 and 2.

[a, b] Different letters within a column indicate differences between protocols at $P<0.05$.

Table 3. Rumen pH, and lactate (mmol/L) and ammonia (mg/L) concentration 9 h after feeding in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet[1] during transition (0-14d) and the first weeks of the fattening period.

		Protocol[1]						P-value		
		1	2	3	Mean	SEM[2]	SEM[3]	Protocol (P)	Day (d)	P*d
Rumen	d 10	6.12	5.91	6.30	6.11	0.186	0.107	0.50	0.82	0.84
pH	d 14	6.11	5.86	6.20	6.05	0.178	0.102			
	d 21	6.42	6.22	6.07	6.24	0.251	0.145			
	d 35	6.20	5.96	6.09	6.08	0.192	0.111			
	d 42	5.98	6.21	6.30	6.16	0.214	0.123			
Lactate	d 10	1.06	1.28	1.31	1.22[a]	0.305	0.176	0.69	<0.001	0.19
(mmol/L)	d 14	1.31	0.99	1.31	1.20[a]	0.292	0.152			
	d 21	0.84	1.26	0.67	0.92[ab]	0.387	0.202			
	d 35	0.52	0.30	0.61	0.48[b]	0.145	0.084			
	d 42	1.21	1.67	0.95	1.28[a]	0.176	0.101			
Ammonia	d 10	117	49	101	89	17.2	9.9	0.21	0.59	0.052
(mg/L)	d 14	63	105	100	89	9.7	5.6			
	d 21	60	102	56	72	15.9	9.2			
	d 35	59	103	113	92	9.5	5.5			
	d 42	86	80	89	85	18.9	10.9			

[1] See Table 2.
[2] Standard error of the mean for comparisons between protocols within days.
[3] Standard error of the mean for comparisons between days within protocols.
[a, b] Different letters within a column indicate differences between sampling days at $P<0.05$.

Table 4. Total volatile fatty acids (VFA) concentration (mmol/L), and molar percentage (mmol/100 mmol) of the main VFA at 9 h after feeding in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet[1] during transition (0-14d) and the first weeks of the fattening period.

		Protocol[1]						P-value		
	Day	1	2	3	Mean	SEM[2]	SEM[3]	Protocol (P)	Day (d)	P*d
VFA	d 10	116	113	99	109[a]	5.8	10.2	0.54	<0.01	0.42
	d 14	124	105	135	121[a]	14.9	25.9			
	d 21	91	92	145	109[a]	10.9	19.0			
	d 30	85	82	79	82[b]	8.2	14.4			
	d 42	92	67	64	74[b]	7.9	13.7			
Acetate	d 10	61.4	59.3	58.4	59.7[a]	2.06	3.57	0.13	<0.001	0.27
	d 14	59.6	56.3	68.5	61.5[a]	3.42	5.94			
	d 21	57.5	57.4	73.2	62.7[a]	3.01	5.22			
	d 30	51.1	55.9	53.4	53.5[b]	1.50	2.61			
	d 42	51.8	44.9	46.6	47.8[c]	1.64	2.85			
Propionate	d 10	16.4	17.2	13.1	18.9[b]	1.14	0.66	0.47	0.001	0.30
	d 14	18.9	16.8	16	17.2[bc]	2.97	1.71			
	d 21	15.5	16.8	11.3	14.5[c]	1.57	0.91			
	d 30	29.3	22.1	17.6	23.1[a]	2.95	1.7			
	d 42	24.6	22.7	26.2	24.5[a]	3.10	1.79			
Butyrate	d 10	14.6	18.0	14.7	15.7	2.94	1.70	0.36	0.45	0.25
	d 14	15.1	19.4	12.0	15.5	4.83	2.79			
	d 21	21.5	18.8	11.4	17.2	3.53	2.04			
	d 30	14.1	16.4	22.7	17.7	2.75	1.59			
	d 42	18.1	24.2	19.2	20.5	3.09	1.79			
Valerate	d 10	1.71	1.21	1.32	1.41[c]	0.204	0.124	0.53	<0.01	0.77
	d 14	2.04	2.16	1.15	1.78[bc]	0.589	0.349			
	d 21	2.37	2.05	0.94	1.79[bc]	0.510	0.295			
	d 30	3.09	2.42	2.45	2.65[a]	0.712	0.412			
	d 42	3.32	3.56	4.03	3.64[a]	0.833	0.483			

[1] See Table 2.
[2] Standard error of the mean for comparisons between diets within days.
[3] Standard error of the mean for comparisons between days.
[a, b, c] Different letters within a column indicate differences between sampling days at $P<0.05$.

671 **Table 5** Effect of adaptation protocol to a high-concentrate diet, and of day of sampling, on the structure of the bacterial community, and on bacterial
672 diversity indexes in the rumen fluid of beef calves during transition (0-14d) and the first weeks of the fattening period.

			Protocol[1] (P)				Day (d)						P-values		
Phylum	Familiy	Genus	1	2	3	SEM	0	10	14	21	30	SEM	P	d	P*d
Actinobacteria			3.52	3.41	3.28	0.13	3.33	3.48	3.41	3.40	3.38	0.147	0.48	0.96	0.71
	Coriobacteriaceae		3.40	3.19	3.09	0.105	3.24	3.23	3.31	3.22	3.14	0.135	0.16	0.92	0.50
		Olsenella	3.11	2.90	2.84	0.113	2.90	3.04	3.00	2.90	2.92	0.146	0.20	0.93	0.32
		Atopobium	1.80	1.43	1.56	0.259	2.11^{a}	1.25^{bc}	1.14^{c}	1.53^{abc}	1.95^{ab}	0.223	0.60	<0.001	0.33
	Bifidobacteriaceae		1.89	2.09	1.65	0.29	0.27^{b}	2.58^{a}	2.29^{a}	2.00^{a}	2.25^{a}	0.317	0.57	<0.001	0.44
		Bifidobacterium	1.71	1.85	1.52	0.243	0.00^{b}	2.56^{a}	2.26^{a}	1.86^{a}	1.78^{a}	0.302	0.64	<0.001	0.38
Bacteroidetes (B)			4.51	4.58	4.61	0.035	4.47	4.60	4.62	4.54	4.59	0.044	0.18	0.12	0.42
	Prevotellaceae		4.32	4.45	4.44	0.038	4.17	4.53	4.55	4.37	4.40	0.047	0.07	<0.001	0.15
		Prevotella	3.22	3.33	3.35	0.08	2.85^{c}	3.93^{a}	3.37^{b}	3.23^{bc}	3.11^{bc}	0.104	0.47	<0.001	0.28
Chloroflexi			1.19	0.93	1.06	0.159	2.41^{a}	0.44^{c}	0.37^{c}	0.80^{bc}	1.28^{b}	0.189	0.54	<0.001	0.50
Fibrobacteres			1.94	2.17	2.45	0.19	2.85	1.99	2.32	1.84	1.93	0.246	0.17	0.03	0.55
	Fibrobacteraceae		1.94	2.17	2.45	0.19	2.85	1.99	2.32	1.84	1.93	0.246	0.17	0.03	0.55
		Fibrobacter	1.82	2.02	2.3	0.186	2.74^{a}	1.85^{ab}	2.12^{ab}	1.69^{b}	1.84^{ab}	0.24	0.18	0.02	0.66
Firmicutes (F)			4.58	4.55	4.50	0.027	4.67^{a}	4.54^{abc}	4.42c	4.58^{ab}	4.51^{bc}	0.035	0.09	<0.001	0.59
	Clostridiaceae		3.85	3.82	3.83	0.053	4.11^{a}	3.86^{b}	3.74^{b}	3.74^{b}	3.70^{b}	0.057	0.90	<0.001	0.89
		Clostridium	2.56	2.74	2.71	0.094	2.90^{ab}	3.01^{a}	2.38^{c}	2.59^{abc}	2.47^{bc}	0.118	0.41	0.0015	0.50
	Eubacteriaceae		3.15	3.07	3.12	0.064	3.60^{a}	3.06^{b}	2.95^{b}	2.95^{b}	3.01^{b}	0.083	0.67	<0.001	0.99
		Eubacterium	2.12	2.10	2.27	0.119	2.59^{a}	2.31^{ab}	2.02^{ab}	2.04^{ab}	1.86^{b}	0.154	0.57	0.01	0.66
	Lachnospiraceae		3.86	3.86	3.84	0.052	3.97	3.91	3.73	3.79	3.86	0.066	0.96	0.10	0.98
		Butyrivibrio	2.00	2.38	2.38	0.209	3.25^{a}	2.47^{ab}	2.10^{b}	1.75^{b}	1.68^{b}	0.210	0.38	<0.001	0.38
		Blautia	1.89	1.70	1.83	0.132	1.97	1.76	1.92	1.75	1.64	0.144	0.60	0.42	0.11
		Catonella	1.67	1.68	1.88	0.109	1.68	2.14	1.66	1.65	1.6	0.141	0.32	0.05	0.17

Coprococcus	1.17	1.31	1.38	0.153	2.71^{a}	1.58^{b}	1.01^{bc}	0.86^{bc}	0.28^{c}	0.198	0.59	<0.001	0.72
Lactonifactor	1.85	1.86	1.69	0.171	1.41	2.13	2.12	1.91	1.43	0.205	0.74	0.02	0.57
Moryella	1.47	1.66	1.84	0.137	1.46	1.55	1.74	1.74	1.78	0.177	0.17	0.63	0.10
Pseudobutyrivibrio	1.15	1.40	1.74	0.154	2.49^{a}	1.55^{b}	1.37^{bc}	1.05^{bc}	0.69^{c}	0.186	0.16	<0.001	0.23
Roseburia	1.01	1.29	1.16	0.241	0.00^{b}	1.75^{a}	1.51^{a}	1.28^{a}	1.18^{a}	0.270	0.70	<0.001	0.20
Lactobacillaceae	2.77	2.55	2.3	0.171	2.43	2.68	2.30	2.77	2.52	0.221	0.16	0.57	0.90
Lactobacillus	1.75	1.36	0.96	0.366	0.00^{b}	1.81^{a}	1.18^{a}	1.89^{a}	1.86^{a}	0.305	0.35	<0.001	0.37
Veillonellaceae	2.68	2.87	2.73	0.100	2.20^{b}	3.25^{a}	3.00^{a}	2.98^{a}	2.37^{b}	0.129	0.38	<0.001	0.95
Selenomonas	2.02	2.37	2.16	0.117	1.72^{b}	2.85^{a}	2.73^{a}	2.57^{a}	1.04^{c}	0.125	0.16	<0.001	0.14
Anaerovibrio	1.55	1.59	1.6	0.134	0.43^{b}	2.38^{a}	1.97^{a}	2.08^{a}	1.03^{b}	0.172	0.96	<0.001	0.93
Ruminococcaceae	3.91	3.89	3.68	0.073	3.65^{b}	3.83^{ab}	3.77^{ab}	4.03^{a}	3.85^{ab}	0.083	0.09	0.03	0.15
Ruminococcus	2.84	2.80	2.74	0.129	3.02^{a}	3.33^{a}	2.84^{a}	2.94^{a}	1.86^{b}	0.162	0.87	<0.001	0.07
Streptococcaceae	1.54	1.89	1.85	0.170	2.52^{a}	1.85^{ab}	1.36^{b}	1.29^{b}	1.79^{a}	0.212	0.11	0.01	0.72
Streptococcus	1.21	1.75	1.47	0.179	2.21^{a}	1.50^{ab}	1.24^{b}	1.09^{b}	1.35^{ab}	0.231	0.51	0.002	0.94
Acidaminococcaceae	2.82	2.74	2.84	0.069	2.50	2.78	2.87	2.81	3.03	0.089	0.99	0.70	0.97
Succiniclasticum	2.45	2.44	2.45	0.101	2.31	2.43	2.57	2.45	2.48	0.130	0.68	<0.001	0.83
Erysipelotrichaceae	2.95	2.92	3.04	0.100	3.46^{a}	2.67^{bc}	2.50^{c}	3.16^{a}	3.07^{ab}	0.112	0.23	0.03	0.21
Sharpea	1.09	0.70	0.55	0.236	0.00^{b}	0.57^{ab}	1.33^{a}	0.97^{ab}	1.03^{ab}	0.304	0.35	0.02	0.79
Proteobacteria (P)	3.22	2.95	3.16	0.128	3.30	2.89	3.26	3.36	2.74	0.154	0.87	0.02	0.24
Succinivibrionaceae	2.18	2.06	2.19	0.204	1.63	2.39	2.55	2.46	1.70	0.252	0.99	0.18	0.64
Ruminobacter	1.00	1.02	0.99	0.307	0.62	1.13	1.54	1.16	0.57	0.335	0.68	0.24	0.83
Succinimonas	0.49	0.25	0.48	0.212	0.00	0.35	0.20	0.78	0.69	0.274	0.83	0.004	0.21
Succinivibrio	1.55	1.63	1.78	0.265	1.44^{ab}	1.92^{a}	1.98^{a}	1.86^{a}	1.06^{b}	0.225	0.96	0.02	0.64
Spirochaetes	1.89	1.88	1.97	0.240	2.69^{a}	1.61^{b}	1.82^{ab}	1.71^{ab}	1.75^{ab}	0.257	0.91	<0.001	0.96
Synergistetes	1.58	1.49	1.59	0.178	2.90^{a}	1.33^{b}	1.16^{b}	1.32^{b}	1.05^{b}	0.217	0.24	<0.001	0.30

Tenericutes	2.03	2.21	2.35	0.133	3.00[a]	1.78[bc]	1.59[c]	2.36[ab]	2.26[bc]	0.171	0.78	<0.001	0.06
Shannon Index	2.22	2.13	2.11	0.122	2.51a	1.85[c]	2.02[bc]	2.20[b]	2.17[b]	0.11	0.80	<0.001	0.04
Richness	31.3	31.2	30.8	0.830	35.7[a]	32.5[b]	30.2[b]	30.5[b]	26.3[c]	1.04	0.92	<.0001	0.63
Ratio F/B	1.09	0.95	1.41	0.172	1.63	0.99	0.89	1.35	0.89	0.222	0.17	0.08	0.69
Ratio P	0.02	0.04	0.08	0.023	0.03	0.02	0.11	0.05	0.02	0.029	0.17	0.16	0.18

673 [1] See Table 2.

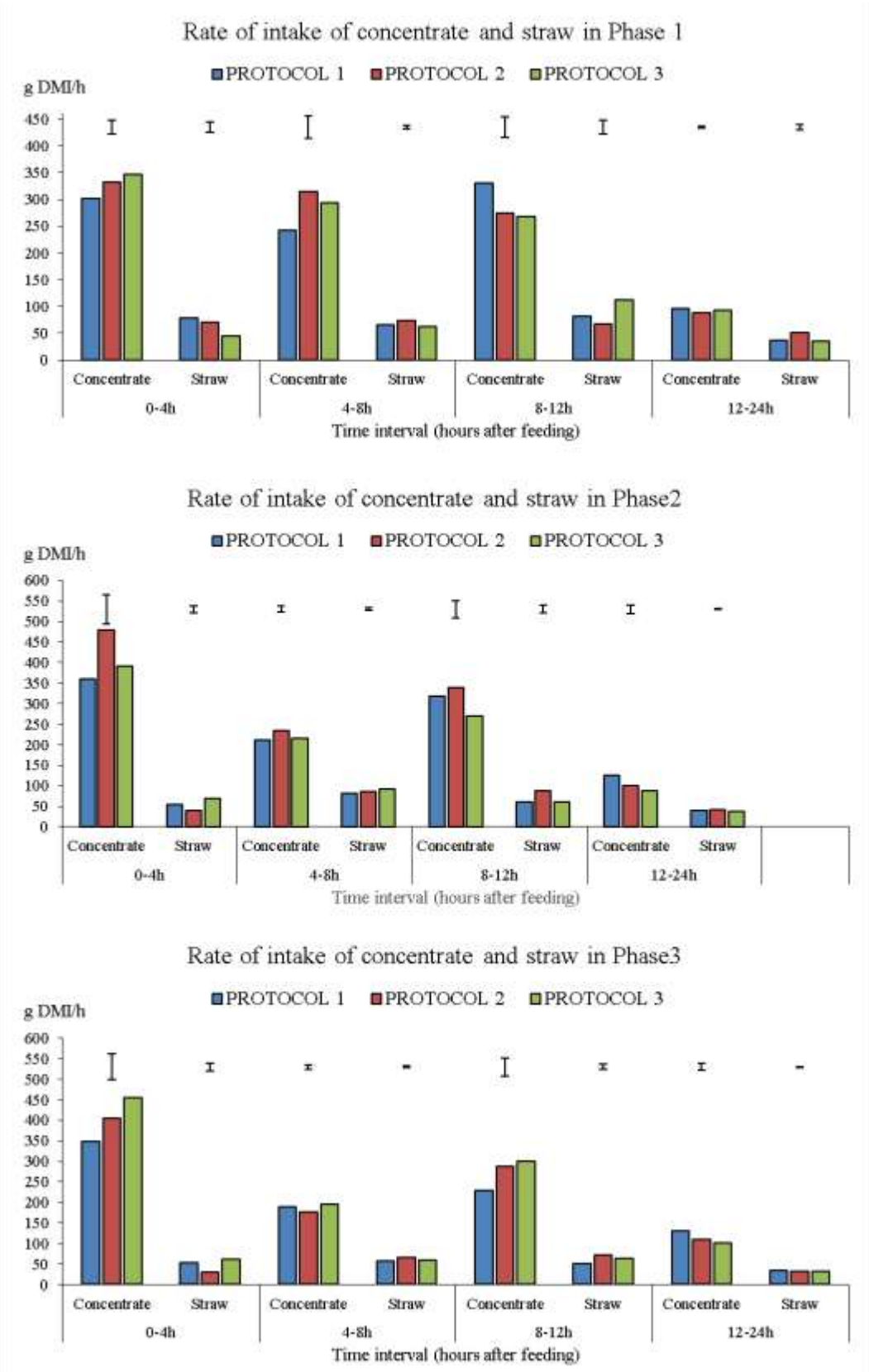
674 The number of reads was normalized to 85,442 reads and log10-transformed (nº reads+1). Only bacteria taxa that represented on average more than
675 0.1% of total sequences are shown. Proteobacteria ratio was calculated as the abundance of Proteobacteria sequences divided by the sum of the
676 abundance of Firmicutes and Bacteroidetes sequences.

677 [a,b,c] Different letters within a row indicate differences between days at $P<0.05$, as obtained using Bonferroni's test.

Table 6. Spearman correlations between bacterial taxa (P: phylum; F: family; G; genus) and rumen fermentation variables of beef calves subjected to different adaptation protocols (P1, P2, P3; See Table 2) to a high-concentrate diet during transition (0-14d) and the first weeks of the fattening period. Only Spearman correlations with r≥0.3 or r ≤-0.3 and P<0.05 are shown (n=60).

	Rumen pH			Lactate			Ammonia			Total VFA			Acetate			Propionate			Butyrate			Valerate			BCFA		
Bacterial taxa	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
P. Actinobacteria	-0.35	-0.42	0.44			-0.31	-0.66		-0.59	0.44		-0.44		0.32			0.38	-0.34		0.46	-0.48	0.51	0.52				-0.49
F. Coriobacteriaceae	-0.33	-0.42	0.45				-0.66		-0.50	0.40									0.43	0.35	-0.52						
G.Olsenella	-0.38	-0.51	0.44			-0.44	-0.55			0.43	0.45		0.33				0.38	-0.36	0.44	0.59	-0.60	0.51	0.63				-0.52
G.Atopobium	0.35	0.39		-0.35	-0.67						-0.38							-0.50		-0.46						-0.57	
F.Bifidobacteriaceae	-0.43	-0.51	-0.43			0.50				0.55	0.64			0.52		0.55	0.58	0.43	0.53	0.50	0.51	0.57	0.50	0.60			
G.Bifidobacterium	-0.43	-0.59	-0.41	0.40	0.34	0.51	-0.45			0.55	0.63	0.34		0.48		0.52	0.56	0.43	0.54	0.54	0.49	0.52	0.56	0.54			
P.Bacteroidetes				0.70				0.58				0.36						0.34			0.48						0.56
F. Prevotellaceae		-0.39	-0.41		0.86	0.31			0.34			0.51				0.52		0.51							0.50		
G.Prevotella			-0.57	0.45	0.67	0.54				0.34		0.57	-0.35		0.43	0.44		0.66	0.44	0.33	0.54	0.34			0.44	0.56	
P.Chloroflexi	0.52	0.51	0.74	-0.68	-0.47	-0.72				-0.34	-0.66	-0.73		-0.50	-0.53	-0.39	-0.47	-0.80	-0.63	-0.59	-0.68	-0.62	-0.54	-0.60	-0.50		
P.Fibrobacteres	0.60	0.63	0.44	-0.51	-0.33	-0.35		0.48			-0.79			-0.58			-0.67	-0.35	-0.58	-0.81	-0.36	-0.65	-0.76	-0.54		-0.42	
F.Fibrobacteraceae	0.60	0.63	0.44	-0.51	-0.33	-0.35	0.48				-0.79			-0.58			-0.67		-0.58	-0.81		-0.65	-0.76	-0.54			
G.Fibrobacter	0.65	0.63	0.53	-0.52	-0.32	-0.38	0.41				-0.78	-0.38		-0.59			-0.64	-0.42	-0.60	-0.77	-0.40	-0.67	-0.75	-0.57		-0.42	
P.Firmicutes	0.40	0.48	0.34		-0.44	-0.31					-0.54			-0.52		-0.73		-0.49			-0.36						
F.Clostridiaceae	0.64	0.55				-0.54					-0.51					-0.64	-0.61			-0.57	-0.54	-0.60	-0.70	-0.68			-0.50
F.Eubacteriaceae	0.59	0.50	0.48	-0.43	-0.63	-0.49				-0.35						-0.49			-0.45		-0.52	-0.52		-0.66	-0.53		-0.51
G.Eubacterium		0.49			-0.57	-0.31	-0.47	-0.38				-0.54			-0.41						-0.58			-0.58	-0.44		-0.66
G.Butyrivibrio	0.82	0.68	0.58	-0.53		-0.31	0.46			-0.49	-0.58	-0.57		-0.32	-0.46	-0.47	-0.64	-0.49	-0.55	-0.63	-0.45	-0.81	-0.70	-0.66			
G.Blautia		0.55					-0.34	0.38			-0.45		0.30				-0.40			-0.52					0.35		
G.Coprococcus	0.60	0.62	0.46	-0.52	-0.43	-0.44	0.32			-0.38	-0.56	-0.52		-0.36	-0.32		-0.58	-0.48		-0.53	-0.47	-0.54	-0.65	-0.63			
G.Lactonifactor	-0.31		-0.50		0.81	0.41		0.43				0.36						0.36	0.39		0.49				0.50	0.69	0.60
G.Pseudobutyrivibrio	0.69	0.50	0.44		-0.45	-0.39				-0.56						-0.51	-0.63	-0.35	-0.57	-0.56	-0.62	-0.75	-0.72	-0.70			
G.Roseburia		-0.63	-0.43	0.71		0.48					0.53	0.34						0.43		0.65	0.53		0.59	0.60			
F.Lactobacillaceae		-0.34									0.59			0.54			0.54			0.51			0.49				
G.Lactobacillus		-0.76	-0.43	0.43							0.61		-0.45	0.34			0.63	0.38	0.44	0.74	0.38	0.53	0.76				
F.Veillonellaceae		-0.44		0.41	0.58								-0.52			-0.45	0.40			0.52					0.61	0.60	
G.Selenomonas					0.68								-0.58					0.52							0.52	0.50	
G.Anaerovibrio				0.51	0.68	0.52							-0.54					0.54	0.37	0.44					0.49	0.71	
F.Ruminococcaceae				0.67	0.47								-0.44	-0.35					0.38						0.53	0.54	
G.Ruminococcus		0.36			0.62	-0.35		0.30	-0.63	-0.34		-0.34				-0.36		-0.37	-0.32								
F.Streptococcaceae	0.73			-0.81	-0.54						-0.54						-0.65		-0.59	-0.57		-0.59	-0.68	-0.58	-0.55		
G.Streptococcus	0.73			-0.59			0.46			-0.45	-0.51			-0.35		-0.34	-0.61		-0.54	-0.49		-0.67	-0.61				
F.Acidaminococcaceae								0.43	0.62							0.50											
G.Succiniclasticum								0.31	0.63					-0.50		0.35										0.59	
F.Erysipelotrichaceae	0.61	0.41	0.61		-0.57	-0.47				-0.65		-0.62			-0.51	-0.61	-0.52	-0.55	-0.66	-0.40	-0.54					-0.57	
G.Sharpea	-0.46	-0.59		0.35			-0.31			0.44	0.47			0.46		0.43						0.68					
P.Spirochaetes	0.59	0.60	0.44	-0.51		-0.37	0.37			-0.53	-0.50	-0.32		-0.32			-0.45	-0.41	-0.57	-0.51		-0.56	-0.60	-0.53		-0.52	
P.Synergistetes	0.61	0.73	0.41	-0.55	-0.37	-0.43				-0.60	-0.54	-0.53		-0.32	-0.40		-0.62	-0.60	-0.48	-0.54		-0.70	-0.65	-0.64			
P.Tenericutes	0.76	0.56	0.57	-0.55		-0.53				-0.57		-0.61			-0.49	-0.52	-0.48	-0.67	-0.77	-0.51		-0.76	-0.60			-0.50	

682 **Figure 1**. Effect of the adaptation protocol of beef calves to a high-concentrate diet (see Table 2) on
683 the rate of intake of concentrates and straw during transition (day 6, Phase1; day 14, Phase 2) and the
684 first week (day 20, Phase 3) of the fattening period. Bars represent the standard error of the mean.
685


Rate of intake of concentrate and straw in Phase 1
PROTOCOL 1
PROTOCOL 2
PROTOCOL 3
g DMI/h
450
400
350
300
250
200
150
100
50
0
Concentrate
Straw
0-4h
4-8h
8-12h
12-24h
Time interval (hours after feeding)
Rate of intake of concentrate and straw in Phase2
PROTOCOL 1
PROTOCOL 2
PROTOCOL 3
g DMI/h
600
550
500
450
400
350
300
250
200
150
100
50
0
Concentrate
Straw
0-4h
4-8h
8-12h
12-24h
Time interval (hours after feeding)
Rate of intake of concentrate and straw in Phase3
PROTOCOL 1
PROTOCOL 2
PROTOCOL 3
g DMI/h
600
550
500
450
400
350
300
250
200
150
100
50
0
Concentrate
Straw
0-4h
4-8h
8-12h
12-24h
Time interval (hours after feeding)