Rumen protozoal dynamics during the transition from milk/grass to high-concentrate based diet in beef calves as affected by the addition of tannins or medium-chain fatty acids.

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Abstract

Changes in rumen protozoal community during adaptation of 7-month-old beef calves from a milk/grass diet to a high-energy diet consisting of cereal-based concentrate plus wheat straw, both given *ad libitum* (diet C), were studied. Eighteen rumen-cannulated Limousine crossbred male calves were randomly assigned to three diets (C; C plus 20 g/kg of a 65:35 chestnut and quebracho tannin extract, T; and C plus 6 g/kg medium-chain fatty acid (MCFA) mixture, M). Fermentation variables were studied, and rumen ciliates were quantified and classified from rumen fluid sampled just before the morning feeding on days 0, 7, 14, 21 and 28 of the experiment. Protozoal population changed over time, but all calves harbored a stable population at the end of the experiment. Diversity decreased on time, and *Entodinium* and *Isotricha* species were the most abundant at the end of the trial. When occurred, defaunation was transient as protozoa were absent from M calves in three occasions on days 7 and 14 but were refaunated thereafter reaching a consistent population. Regardless the diet, proportions of *Isotricha* spp. and *Dasytricha ruminantium*, *Epidinium* spp. and Subfamily Diplodiniinae*,* decreased throughout the experiment, whereas *Entodinium* spp. increased reaching 0.93 of total protozoa. *Dasytricha* and protozoa from Subfamily Diplodiniinae disappeared from the rumen of all calves at the end of the study, except for *Polyplastron multivesiculatum.* Rumen variables were not affected by the addition of additives ($P > 0.05$). Rumen pH decreased across the study ($P < 0.001$) but did not reach acidotic values despite the high fermentability of the diet. Higher levels of lactate were observed on C calves at d14, but not in other dates (diet by day interaction, $P = 0.03$). Addition of tannin extract did not affect protozoal concentration and diversity, but MCFA might even lead to a transient defaunation. Transition to a high-concentrate diet does not necessarily reduce initial protozoal concentration, but diversity is affected towards a major proportion of *Entodinium* and, to a lesser extent, *Isotricha*. Dietary shift from weaning to a high-concentrate ration promotes a less diverse rumen protozoal population, but a high concentration is still maintained thereafter.

Keywords: calves, transition, rumen protozoa, tannins, medium-chain fatty acids

Abbreviations: ADF, acid detergent fibre; NDF, neutral detergent fibre; BCFA, branched-chain fatty acids, CP, crude protein; DM, dry matter; EE, ether extract; MCFA, medium-chain fatty acids; OM, organic matter; VFA, volatile fatty acids.

1. Introduction

Under the conventional intensive beef production in some Mediterranean countries, calves are abruptly weaned and immediately switched from a milk and/or high-forage regime to a high-concentrate diet. Then, the enhanced activity of rumen microbiota with rapid fermentation of starch to organic acids (volatile fatty acids -VFA- and lactate) may lead to an accumulation of fermentation products if the rate of acid production exceeds acid removal from the rumen. This ruminal imbalance increases the risk of a marked drop in rumen pH below 5.0 or 5.6, level generally considered as threshold for acute or subacute ruminal acidosis, respectively (Owens et al., 1998; Krause and Oetzel, 2006), although pH is not always a good indicator of acidosis (Villot et al., 2018). In such situation, it is of great interest to slow down the rate of fermentation and maintain the rumen pH above the mentioned threshold. The use of feed additives to modulate the rate and extent of the microbial fermentation is common to mitigate the consequences of dietary shift during transition period, which comprises the first 14-21d from the arrival of the newly weaned calves to the feedlot. Tannins (Baah et al., 2007; Amanzougarene et al., 2018) and certain medium-chain fatty acids (MCFA) (Matsumoto et al., 1991; Hristov et al., 2004) have been assessed for their potential to interact with rumen microbiota to modulate ruminal fermentation and improve nutrient utilization in ruminants.

Rumen protozoal population plays an important role in nutrient metabolism and in the rumen environmental balance (Newbold et al., 2015). They contribute to slow down rumen starch digestion by ingesting starch granules and store them as amylopectin, at a fast rate (up to 0.77 mg/mg protein per minute), which may imply a 0.35 sequestration and 0.20 metabolisation of the daily starch intake of sheep (Coleman, 1992). This reduces starch availability for bacteria and thus prevents its rapid fermentation, which otherwise might promote a decrease in rumen pH (Mackie et al., 1978; Mendoza et al., 1993). In addition, entodiniomorphid protozoa actively

contribute to the ruminal metabolism of lactic acid (Newbold et al., 1987), reducing the risk of rumen pH to decline. Thus, a remarkable stabilizing rumen pH effect has been associated to the presence of protozoa (Williams and Coleman, 1992; Jouany and Ushida, 1999), which is especially critical when newly weaned calves are transitioned to high-concentrate, starch-rich diets (Brown et al., 2006). Numerous observations in cattle fed high-concentrate diets have reported a reduction or even absence of rumen ciliate protozoa, as well as a reduction of protozoal diversity to a maximum proportion of *Entodinium* species (Eadie et al., 1970; Lyle et al., 1981). However, other studies have found a considerable protozoa population in feedlot cattle (Towne et al., 1990; Franzolin and Dehority, 1996). In addition, protozoal community varies widely among hosts and their survival in cattle fed high concentrate diets *ad libitum* depends on other factors such as the roughage level, length of time on feed or inclusion of additives, and fluctuates in response to changes in ruminal conditions (Dehority, 2003). In this regard, the potential benefit of an additive on rumen fermentation could synergically or antagonistically interact with the presence and role of protozoa.

We hypothesized that calves that had been abruptly weaned and introduced to a high-concentrate diet have reduced protozoal population in a sense that can affect rumen fermentation towards an acidification of the environment and the risk of acidosis. Therefore, the objective of this work was to determine the quantitative and qualitative effect of the addition of tannins and MCFA after the transition from milk and grass diet to a high-concentrate ration on ciliate protozoa and on the relationship between protozoa and rumen environmental conditions.

2. Materials and methods

Animal care, handling and surgical procedures were approved by the Ethics Committee of the University of Zaragoza. 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. Eighteen 7-month old (212 ± 27.0 kg average body weight and 224 ± 54.3 d) Limousine crossbreed male calves from different Spanish locations (seven from Extremadura, six from Castilla y León, four from

Cantabria and one from Asturias) were used. Animals were reared with their dams on pasture and allowed to suckle freely while they were grazing. Then, calves were abruptly weaned and transported to the Servicio de Experimentación Animal (University of Zaragoza) where they received grass hay (g/kg: organic matter, OM 901; crude protein, CP 112; neutral detergent fibre, NDF 571; acid detergent fibre, ADF 343; lignin 45) *ad libitum* for their adaptation to the farm environmental conditions. Animals were individually housed in a 3.4 x 3.4 m pens provided with slatted concrete floor, automatic water dispenser and separate troughs for concentrate and roughage. One week after their arrival, calves were fistulated in the dorsal sac of the rumen with a 150 mm long, 15 mm I.D. permanent cannula. Animals were allowed to recover from surgery for two weeks, and therefore the experiment began three weeks after the arrival of the animals.

2.1. Experimental design

The experiment was carried out for 28 days. Calves were abruptly switched from an all forage diet to a ration consisting of a cereal and soybean meal-based concentrate (Table 1) in meal form (ground through a 3.5 mm sieve) plus wheat straw (offered in long form), both given *ad libitum*. The three dietary treatments were: a non-supplemented diet, considered as a control (C); C plus 20 g/kg (as fed) of a commercial 65:35 chestnut (*Castanea* spp.) and quebracho (*Schinopsis* spp.) tannin extract containing over 0.65 of tannins (Vinitanon, Agrovin, Spain; T); and C plus 6 g/kg (as fed) of a commercial mixture of medium-chain fatty acids (OptimaPLUS, Nutrika, Belgium; M). Analyzed proportions of fatty acids in M were: caproic acid, C_6, 0.10; caprylic acid, C_8, 0.20; capric, C_{10}, 0.20; and lauric acid, C_{12}, 0.50. Doses of additives were selected according to the statements of the manufacturer of the extracts, and were previously studied in two *in vitro* trials (Amanzougarene et al., 2017; 2018). Six calves were randomly allocated to each experimental diet, ensuring homogeneous average weight and standard deviation per group. Concentrate was offered *ad libitum* once a day at 08:00 h, with the ration daily adjusted to ensure at minimum 0.10 refusals. Straw was offered three times daily to ensure *ad libitum* access, intending to keep at least 0.5 kg straw at any time in the feeder. The amount of concentrate and

121 straw offered and refused were daily recorded throughout the experiment. Weekly samples of
122 both concentrate and straw were taken for chemical analysis.

123 On days 0, 7, 14, 21 and 28, representative volumes (200 mL) of rumen fluid were
124 sampled from each calf at 08:00 h, just before feed distribution. Two mL subsamples were taken
125 with a wide-mouth (3 mm) pipette, diluted in an equal volume of 18.5% formaldehyde and kept
126 at room temperature until the protozoal counts were performed. Rumen pH was measured with a
127 portable pH-meter (Seven2GO, Mettler-Toledo AG, Schwerzenbach, Switzerland); then, rumen
128 fluid was strained through a 1 mm metal mesh. Three 4 mL aliquots were sampled in duplicate
129 for ammonia (in 4 mL 0.1 N HCl), lactate and volatile fatty acids (VFA; in 1 mL solution made
130 up with 2% orthophosphoric acid and 0.2% 4-methyl valeric acid) analysis. Samples were frozen
131 (-20 ºC) until further analyses.
132
133 *2.2. Chemical and microbiological analysis*

134 Concentrate and straw refusals were collected daily and fortnightly, respectively, pooled
135 on an animal basis and weighed weekly and fortnightly. Dry matter (DM) of feeds and refusals
136 was determined by oven drying at 65ºC to a constant weight. Feeds were analysed following the
137 procedures of AOAC (2005) for their OM (ref. 942.05), CP (ref. 976.05) and ether extract (EE;
138 ref. 2003.05) content. Concentration of aNDF was analysed using an Ankom 200 Fiber Analyzer
139 (Ankom Technology, New York) as described by Mertens (2002); both ADF and lignin in
140 sulphuric acid were determined as described by Robertson and Van Soest (1981). The aNDF is
141 expressed exclusive of residual ashes, and α–amylase was used in the analysis; sodium sulphite
142 was not used. Total starch content was determined enzymatically in samples ground to 0.5 mm
143 by using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland).
144 The concentration of VFA in rumen samples, and the concentration of individual MCFA in the
145 additive, were determined by gas chromatography in an Agilent 6890 apparatus (Agilent
146 Technologies España S.L., Madrid, Spain) fitted with a capillary column (Model HP-FFAP
147 polyethylene glycol TPA-treated, 30m x530µm I.D. x1µm film thickness). Ammonia and total

148 lactate concentrations were measured colorimetrically following the methods proposed by
149 Chaney and Marbach (1962), and Barker and Summerson (1941), respectively.
150 Rumen protozoal quantification was performed by optical observation using a 10x
151 eyepiece with x10 and x40 objectives through a microscope (Axiolab, Carl Zeiss Jena, Germany),
152 in a Sedgewick-Rafter counting chamber following the procedure of Dehority (1993), except for
153 those cases with extremely low protozoal numbers, when the entire chamber was counted. Genera
154 and some species were identified as outlined by Dehority (1993), and Ogimoto and Imai (1981).
155 During each counting, the numbers of different genera in the protozoal population were recorded,
156 identified and grouped into the genera *Isotricha* and *Dasytricha* from the Family Isotrichidae;
157 genus *Epidinium* from the Subfamily Ophryoscolecinae; genus *Entodinium* from the Subfamily
158 Entodiniinae; and genera *Diplodinium, Metadinium, Eudiplodinium, Enoploplastron,*
159 *Ostracodinium* and *Polyplastron* from the Subfamily Diplodiniinae. Total protozoal and groups
160 concentration for each calf were calculated and transformed into logarithmic basis (log_{10}/mL)
161 before being subjected to statistical analysis.
162
163 *2.3. Statistical Analysis*
164 All data were analyzed by ANOVA using the MIXED procedure of SAS (v9.4), with
165 animal considered as the experimental unit. Analysis of concentrate or straw DM intake, protozoal
166 concentration and rumen fermentation variables were carried out as a repeated measures design
167 considering the experimental diet, sampling day and their interaction as fixed effects, and animal
168 within diet as random effect. Sampling day was considered as the repeated measure. Initial age
169 and body weight were included in the model as covariates for analysis of DM intake. Polynomial
170 (linear, quadratic and cubic) contrasts were established using the CONTRAST statement of SAS.
171 Differences between means were calculated using the 'PDIFF' option in the 'LSMEANS'
172 statement of the MIXED procedure. The level of significance was set at $P \leq 0.05$ and trends were
173 stated at $P \leq 0.10$ unless otherwise indicated.
174 Spearman's correlation coefficients between variables were calculated using the CORR
175 procedure. Absence of total protozoa or any protozoal group was considered as zero in the

176 statistical analyses. Results for the proportions of protozoal groups are presented as arithmetic
177 means with their standard deviation.
178
179 **3. Results**
180 *3.1. Feed intake and rumen fermentation variables*
181 No treatment differences were found in concentrate or straw intakes (Table 2) throughout
182 the experiment ($P > 0.05$); although they respectively increased and decreased over time ($P =$
183 0.01). Consequently, proportion of concentrate increased ($P < 0.001$) from d14 to 28, although
184 again no differences among treatments were found ($P > 0.05$).
185 Even though it was not the main objective of this work, fermentation variables were
186 measured weekly for characterizing rumen environmental conditions and establishing potential
187 relationships that might help describing protozoal pattern. Therefore, only means for the main
188 effects are presented in Table 3, and the diet x time interaction (significant only for lactate
189 concentration) is commented below.
190 Rumen pH was unaffected by diet ($P = 0.16$) but decreased linearly over time ($P < 0.001$).
191 Total VFA concentration was higher ($P = 0.035$) in calves given diet C than in those given diet T,
192 with intermediate values for animals on M diet. Total VFA increased on d28 ($P < 0.001$) with
193 respect to the previous dates. Molar VFA proportions were not affected by diet ($P > 0.10$), but all
194 of them except propionate ($P = 0.13$) varied among sampling dates ($P < 0.05$). Except for d28,
195 butyrate, valerate and branched-chain fatty acids (BCFA) proportions increased at the expense of
196 acetate. Even though there were no differences among diets on rumen lactate concentration on
197 days 0 and 7, values in C calves became the highest on d14 with no differences between T and
198 M, and those differences disappeared thereafter (interaction diet x time, $P = 0.033$). Ruminal
199 ammonia concentration was unaffected by diet or by date ($P > 0.10$).
200
201 *3.2. Protozoal counts*
202 Overall, average protozoal concentration was 5.82 log cells/mL although fluctuations
203 were observed throughout the experimental period, both within and between calves (coefficient

204 of variation 0.24). Individually, eight calves (four from diet C, three from T and one from M)
205 maintained a stable protozoal population on time with a concentration over 5 log cells/mL,
206 whereas another two animals (both from diet M) were defaunated (absent of protozoa) on d7 and
207 d14, but were recolonized after one or two weeks reaching consistent concentrations thereafter.
208 The interaction between diet and sampling day showed a trend for an effect on protozoal
209 concentration across the trial (P= 0.078; Figure 1); thus, on d7 and d14, animals on diet M had a
210 lower protozoal concentration whereas no differences were found between the other diets (6.11,
211 6.34 and 4.12 log cells/mLon d7, and 6.63, 6.52 and 4.82 log cells/mL on d14, for diets C, T and
212 M, respectively). Protozoal concentration in group M followed a cubic response (P= 0.018) across
213 the trial; the marked drop in concentration found in this group on d7 and d14 was mainly due to
214 the aforementioned defaunation of two animals, and to low concentrations in another two calves
215 (2.82 log cells/mL in one animal on d7, and 3.06 log cells/mL in the other calf on d14). A low
216 rumen protozoal concentration (below 4 log cells/mL) was also observed in one animal from diet
217 T on d21, and in one animal per treatment on d28.

218 The concentration pattern of the different protozoal groups throughout the study is shown
219 in Figure 2. Differences in concentrations of *Isotricha* among diets were observed over time
220 (interaction diet x sampling date, P= 0.04); thus, in C calves tended to follow a quadratic
221 evolution (P= 0.09), in T calves it showed a linear decrease (P< 0.001) and in M a sharp quadratic
222 decrease (P= 0.03). In contrast, concentration of *Dasytricha*, which on d0 showed a proportion
223 over 0.20 of total protozoa in six out of 18 calves, was quadratically (P= 0.001) reduced, and
224 subsequently disappeared from the rumen of all calves from d14 (C), d21 (M) and d28 (T). No
225 dietary differences were observed for *Dasytricha* concentration. Despite *Entodinium* spp.
226 concentration across diets behaved similarly than that for total protozoa on d7 and d14,
227 *Entodinium* spp. were not affected by diet (P= 0.16) and maintained average concentrations
228 between 5.35 and 6.63 log cells/mL. Despite its constant concentration throughout the experiment
229 (P= 0.70), proportion of *Entodinium* widely differed among calves, ranging from below 0.40 in
230 three calves to over 0.70 in another four animals on d0, and increasing to over 0.90 of total
231 protozoa from d7 onwards (Table 4). Initially (d0), the genus *Epidinium* was detected in only six

232 animals in low (from 0.83 to 2.67 log cells/mL) but relatively constant concentrations (Figure 2)
233 and remained present in four out of 18 calves on d28 (Table 4). No effects of diet (P= 0.45) or
234 sampling day (P= 0.20) were found. Protozoa from six genera of the Subfamily Diplodiniinae
235 (*Diplodinium, Metadinium, Eudiplodinium, Enoploplastron, Ostracodinium* and *Polyplastron)*
236 were detected in all animals at the beginning of the experiment, with an average concentration of
237 5.11 log cells/mL. This group of protozoa was not affected by diet (P= 0.14) but changed over
238 time (average values of 1.65, 2.18, 2.24 and 1.77 log cells/mL on d7, 14, 21 and 28, respectively;
239 P< 0.001), remaining present in only seven out of the 18 calves at the end of the trial (Table 4).
240 It is worth mentioning that, in most cases, the presence of protozoa of this group was supported
241 by the genera *Polyplastron* and *Eudiplodinium*, whereas *Metadinium* and *Enoploplastron*
242 completely disappeared from d7. At the end of the study (d28), only *Polyplastron* (in five calves),
243 *Eudiplodinium* (in three calves) and *Diplodinium* (in one calf) were detected.
244 A wide and diverse protozoal community was observed in weaned calves at the start of
245 the experiment, with protozoal diversity (Types A and B; Eadie, 1962) partly depending on the
246 origin of calves. The four calves from Cantabria had a Type B population including *Entodinium*,
247 holotrichs (*Isotricha* and *Dasytricha*), *Diplodinium* and *Ostracodinium* species, and
248 *Eudiplodinium* and/or *Epidinium* together or separately, whereas the calf from Asturias (which is
249 a neighbor region) had Type A protozoa, containing *Entodinium* and holotrichs species plus
250 *Polyplastron multivesiculatum* as the predominant large entodiniomorph. Moreover, calves from
251 Extremadura and from Castilla y León harbored either Type A or Type B protozoa. Although
252 most of the initially observed protozoal species were present in all animals on d0, some degree of
253 specificity occurred, and *Metadinium affine* and *Epidinium caudatum* were only present in the
254 seven calves from Extremadura, whereas *Entodinium rostratum* was present in all animals except
255 in those from this region; similarly, *Ostracodinium mammosum* and *Eudiplodinium dilobum* were
256 found in the six animals from Castilla y León, and *Epidinium parvicaudatum* was only present in
257 the four animals from Cantabria.
258 Even though species description was not the main objective of this work, up to 26
259 protozoal species were identified at the start of the experiment (d0). *Isotricha* spp. (mostly *I.*

prostoma) was present in all animals, and *Dasytricha ruminantium* was detected in 17 calves. Six *Entodinium* species were identified (the number of calves harboring those species is showed in brackets): *E. nanellum* (18), *E. longinucleatum* (11), *E. exiguum* (10), *E. dubardi* (10), *E. caudatum* (9) and *E. rostratum* (7), as well as three *Epidinium species*: *E. eucaudatum* (6), *E. caudatum* (3) and *E. parvicaudatum* (3). Among the Subfamily Diplodiniinae, three *Diplodinium* (*D. dentatum*, 5; *D. monolobosum,* 4; and *D. lobatum,* 3); three *Eudiplodinium* (*E. bovis,* 7; *E. maggi,* 7; and *E. dilobum,* 5); six *Ostracodinium* (*O. gracile,* 7; *O. obtusum,* 2; *O. rugoloricatum,* 2; *O. mammosum,* 2; and *O. trivesiculatum,* 1); two *Metadinium* (*M. medium,* 4 and *M. affine,* 3); *Enoploplastron triloricatum* (4) and *Polyplastron multivesiculatum* (12) were observed.

Significant correlations were observed between concentrations of some protozoal groups and some rumen fermentation variables (Table 5). No relationships were established for *Dasytricha* and *Epidinium* because of the low number of data pairs (31 and 24, respectively, out of 90 possible counts). Even though some relationships were significant ($P< 0.05$), the level of significance for correlation analyses was considered at $P< 0.01$ to discern meaningful correlations between variables. Spearman's correlation coefficients were relatively low, because of the major importance of the bacterial role in fermentation. Total protozoa and *Entodinium* were positively correlated with butyrate, BCFA, lactate (not for *Entodinium*) and ammonia concentration ($P< 0.01$). Diplodiniinae spp. were positively correlated with pH ($P< 0.001$), and negatively with butyrate, valerate and lactate ($P< 0.01$). Similarly, a relationship between the proportion of straw in the ration and total protozoa concentration was found ($r= 0.43$, $P< 0.01$).

4. Discussion

When young ruminants are abruptly switched from a milk plus forage to a high-grain diet, the protozoal population markedly changes. If the dietary proportion of concentrate exceeds 0.60 of the total diet, a subsequent depression in ruminal pH leads to a decrease in the protozoal concentrations and diversity (Mackie et al., 1978) that eventually might result in the rumen defaunation (Eadie et al., 1970; Lyle et al., 1981). However, some studies have demonstrated that feedlot cattle fed high- or all-concentrate diets *ad libitum* even possess consistent protozoal

concentrations (Franzolin and Dehority, 1996; Hristov et al., 2001), suggesting that a microbial adaptation to ruminal conditions occurs progressively, this response depending on individual variability. When rumen is under development, the microbial population might be more affected to dietary changes (Yáñez-Ruiz et al., 2015) but animals with a fully functional rumen because of age and forage intake, such those in the present study, might be more prepared to withstand metabolic challenges after an abrupt weaning.

Despite the inherent problems to define a representative pH value for rumen environment (Villot et al., 2018), rumen pH has been assumed to be a key factor in the establishment and maintenance of the ruminal protozoa population, and defaunation in cattle consuming grain-rich diets has been ascribed to the low pH (5.5 and below) caused by these diets. However, Lyle et al. (1981) found that protozoa were eliminated in steers despite rumen pH values between 5.7 - 6.0, whereas Franzolin and Dehority (1996) found consistent protozoa population in steers that had rumen pH values lower than 5.7 for 12 h/d. Therefore, the pH and the time that pH values remain low are not the only factors responsible for a marked decrease in protozoal population or even defaunation as pointed out by Franzolin and Dehority (1996). In the present study, ruminal pH was measured once a day before the morning feeding, so we could not monitor diurnal post feeding rumen pH variations. However, rumen pH values recorded 6 h post-feeding in a parallel study (data not shown) were below 6.05 only in 19 out of 72 measurements (18 calves in four different dates from d7 to d28) despite the high fermentability of the diet and the sudden dietary shift. Throughout the experiment, in three cases, M calves were defaunated or harbored a low protozoal concentration (below 3 log cells/mL) on d7 and d14. Ruminal pH on that sampling date in those animals was 6.22, 7.26 and 7.08, respectively, values well over the pH considered as threshold for growth of ruminal protozoa. Therefore, protozoal changes cannot be explained because of rumen pH, which remained quite stable. Instead, it might be due to the toxic effect of MCFA on rumen protozoa, as discussed below. However, those aforementioned animals recovered their population in subsequent dates, as reported by Towne et al. (1990). Refaunation could have been endogenous, with protozoa surviving in the rumen at undetectable levels or migrating from the omasum (Towne and Nagaraja, 1990), or exogenous, by contact from a

faunated neighbor calf (Eadie and Mann, 1970). Defaunation or large reductions in ruminal fauna are generally associated with physical and chemical modifications of the rumen environment (Williams and Coleman, 1992), being a decrease in ruminal ammonia the most consistent effect of defaunation, because of a decreased proteolytic activity and bacterial lysis after elimination of protozoa (Jouany et al., 1988). In our work, positive correlations between total protozoal concentration and rumen ammonia and BCFA, as an index of proteolysis, were observed (Table 5). The positive correlation between total protozoa and lactate can be related to the normal lactate production derived from starch utilization, since no high lactate concentration or low pH were found. On the contrary, protozoa in the Subfamily Diplodiniinae, which are mainly fibrolytic, showed a negative correlation with lactate and a positive relationship with pH suggesting that this group is sensitive to high concentrations of lactate and the subsequent low pH. Similarly, the absence of protozoa is associated in the meta-analysis conducted by Newbold et al. (2015) with a clear reduction of ammonia concentration and butyrate, but no major effect on lactate concentration and a lack of response on pH or VFA concentration.

In the present experiment, maintenance of a stable concentration of total protozoa might be associated with a balanced rumen fermentation, by preventing a low ruminal pH. The symbiotic relationship between protozoa and host is particularly important during the transition from high-forage diets to rations high in readily fermentable carbohydrates (Brown et al., 2006), through the protozoal ability to uptake readily fermentable starch granules, reducing the rate and extent of digestion in the rumen (Mendoza et al., 1993; Fondevila and Dehority, 2001). For instance, holotrichs rapidly assimilate sugars, fermenting and storing them as amylopectin, and entodiniomorphs actively ingest starch granules and have an important role in the lactic acid clearance in the rumen (Newbold et al., 1987). This provides the rumen with buffering capacity and prevents the detrimental effect that might result from an overload of fermentable material with the subsequent over-acidification of the environment.

Even though diversity decreased throughout the experiment, *Entodinium* was not the only genus present, as holotrich protozoa (*Isotricha*) and *Polyplastron* were also present in some calves on day 28. Granja-Salcedo et al. (2016) observed a stable rumen protozoal concentration over 6.0

log cells/mL in steers given concentrate proportions increasing from 0.30 to 0.80, with *Entodinium* spp. as 0.99 of total population and only minor presence (around 3.0 log cells/mL) of *Eudiplodinium* and *Eremoplastron* with the highest level of concentrate, averaging a rumen pH of 6.0. Our results support those studies, as we observed a consistent and relatively stable protozoal population after 7 days with a high (0.83 to 0.90 of total intake) concentrate ration.

The effects of tannins on rumen protozoa are controversial. Positive (Vasta et al., 2010), negative (Makkar et al., 1995; Hristov et al., 2003) or absence (Śliwiński et al., 2002; Benchaar et al., 2008) of effects on the protozoal population have been reported. Moreover, Jayanegara et al. (2012) revealed in their meta-analysis that there is no clear relationship between dietary tannins and rumen protozoa. In fact, comparison among studies should be made with caution since the effects depend on supplementation level, origin, molecular weights and type (condensed *vs.* hydrolysable) of tannins (Patra and Saxena, 2009; Saminathan et al., 2017). Besides, the effect of tannins on protozoal dynamics under a high-concentrate feeding regime is not well documented, since most studies have been conducted in animals given all-forage or mixed forage-grain diets, or under *in vitro* conditions, where protozoal survival is reduced and the applicability to *in vivo* conditions is limited. Most of the information in the literature is about the effect of condensed tannins (i.e. from quebracho or *Leucaena leucocephala*), while the effect of hydrolysable tannins (i.e. from chestnut or tannic acid) on rumen protozoa *in vivo* is scarce. We used a 65:35 commercial mixture of chestnut and quebracho tannins included at 20 g/kg of concentrate, and we found no effect of tannins on any of the studied variables. Carulla et al. (2005) reported a reduction in holotrich protozoa in a forage-based diet with the addition of 40 g/kg DM condensed tannins extract from *Acacia* in sheep, whereas total protozoal population was unaffected. On the contrary, an increase in total protozoa was observed in lambs supplemented with 100 g/kg of quebracho tannins (Vasta et al., 2010). No effects on holotrichid or entodiniomorphid protozoa were observed by Piñeiro-Vazquez et al. (2018) after two weeks of adaptation to a diet including *L. leucocephala* (containing 20 g/kg of condensed tannins), and similar results were observed by Śliwiński et al. (2002) in growing lambs fed a 1:1 hay and concentrate diet supplemented with the same proportion of chestnut tannins extract. However, Saminathan et al. (2017) reported an

overall decrease of protozoa population (increase of *Diplodinium* and a decrease of *Entodinium, Eudiplodinium, Polyplastron* and *Metadinium* proportions) measured by molecular techniques, but results were obtained after only 24 h of *in vitro* culture of bovine rumen fluid with a forage added with 30 g/kg of condensed tannins from *L. leucocephala*. Also *in vitro*, Makkar et al. (1995) observed a general decrease in protozoa, but of higher magnitude on holotrichs, with quebracho tannins *in vitro* (0.1-0.4 g/L). In terms of protozoal types, in the present study no major effect of tannins addition compared to the control diet was observed on concentration of *Entodinium* or *Dasytricha* (disappearance of the latter was retarded in two weeks), and a slight attenuation of changes in *Epidinium* and Diplodiniinae groups was apparent. However, the decrease in *Isotricha* was linear and its defaunation was observed in four calves given diet T. Based on these results, there is no apparent effect of tannins on protozoal community or on rumen fermentation variables.

The MCFA are commonly used as additives to modulate rumen microbiota, as they have a selective effect on certain microorganisms (Henderson, 1973). In their meta-analysis, Guyader et al. (2014) reported a decrease of protozoa with MCFA addition. *In vivo*, Faciola and Broderick (2014) reported a 0.40 decrease of rumen protozoa with 13 g/kg DM of lauric acid (C_{12}) in lactating dairy cows, and Matsumoto et al. (1991) observed defaunation after 2 days with either 50 g/kg (as fed) of C_{10} or C_{12}, and reductions to around 0.10 with the same proportion of C_8 in goats. Similarly, Machmüller and Kreuzer (1999) reported a reduction in the protozoal population in sheep after 21 days with a dietary inclusion of 35 g/kg of coconut oil, which resulted in average intakes of 5.0, 2.8 and 16.7 g/d of C_8, C_{10} and C_{12}, respectively. In another experiment, Jordan et al. (2006) found reduced protozoal numbers in beef heifers supplemented with 250 g/d of coconut oil. In an *in vitro* experiment, Hristov et al. (2004) reported a consistent inhibition with caprylic (C_8) at 1.25 g/L, and elimination of protozoa with capric (C_{10}) and lauric (C_{12}) at doses ranging from 0.6 to 2.5 g/L, whereas no effect of caproic (C_6) acid on total protozoal numbers was found. Despite the high variability among studies regarding feeding conditions and experimental approach, it is clear that MCFA possess a strong antiprotozoal activity. It is worth considering that doses used in the mentioned studies were much higher than that used in the present work (6 g/kg of concentrate). In any case, the two animals that were defaunated on d7, as well as those in

400 which low protozoal counts were found, were given diet M, so such effect could be attributed to
401 the inclusion of MCFA even at such a low dose. Regarding the effect of MCFA on the dynamics
402 of protozoal types, there were no major differences (except for *Isotricha*) between M and the other
403 diets (Figure 2). Besides, defaunation was transient and protozoal concentration was recovered,
404 suggesting an adaptation of the ruminal microbiota to the additive. Some studies have reported
405 that microbial populations exhibit a remarkable resilience and ability to adapt rapidly to a wide
406 variety of antimicrobial agents (Newbold et al., 1977; Baah et al., 2007). Newbold et al. (1977)
407 suggested that it is not the protozoal population *per se* that is resistant to the antiprotozoal agent,
408 but certain bacterial population is capable of degrading the antiprotozoal component. Thus, the
409 lack of effect of diets M and T on protozoal population found in the present study might probably
410 be due to the adaptation of the microbiota to the tested additives.
411
412 **5. Conclusions**
413 A relatively high protozoal concentration was found in cattle fed a 0.88 concentrate ration
414 given *ad libitum* for four weeks after weaning from a milk/forage diet. However, diversity was
415 affected, and some protozoal types such as *Dasytricha* and most of Subfamily Diplodiniinae
416 (except for *P. multivesiculatum* and *Eudiplodinium* spp. in some cases) disappeared from the
417 rumen of calves. The presence of protozoa was probably important to maintain stable rumen
418 conditions avoiding the risk of acidosis, although it increased proteolysis and ammonia
419 concentration. The lack of a major antiprotozoal effect of tannins and MCFA used as additives
420 was probably due to a microbial adaptation and resilience capacity of the microbial population
421 and, for the latter, to the low dose used.
422
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573

574 **Table 1.** Ingredient and chemical composition of diets: a non-supplemented diet (C); C plus 20
575 g/kg of a commercial 65:35 chestnut and quebracho tannin extract containing over 0.65 of tannins
576 (T); and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids (M). Composition of
577 wheat straw is also included.

	Concentrates			Wheat
	C	**T**	**M**	**straw**
Ingredients (as fed basis), g/kg				
Barley	590	572	587	
Maize	151	148	150	
Corn gluten feed (200 g CP/kg)	57	56	57	
Soybean meal (470 g CP/kg)	172	169	171	
Palm oil	9.3	9.1	9.3	
Urea	0.6	0.6	0.6	
Calcium carbonate	8.5	8.3	8.4	
Dicalcium phosphate	5.0	4.9	5.0	
Sodium chloride	5.0	4.9	5.0	
Vitamin-mineral premix	2.0	2.0	2.0	
OptimaPLUS (Nutrika, Belgium)	-	-	6	
Vinitanon (Agrovin, Spain)	-	20	-	
Nutrient composition (g/kg DM)				
OM	945	947	947	937
CP	164	168	169	22
EE	33	29	32	12
Starch	471	484	500	5
NDF	155	149	157	814
ADF	49	46	47	473
Lignin	4	3	3	42

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre

578

Table 2. Average dry matter intake (kg/d) of concentrate (from 0 to 28 days) and straw (from 0 to 28 days), and concentrate proportion of total dry matter intake, by beef calves given a non-supplemented diet (C); C plus 20 g/kg of a chestnut and quebracho tannin extract (T); and C plus 6 g/kg of a mixture of medium-chain fatty acids (M) during the adaptation period to a high-concentrate feeding.

	Concentrate (kg DM/d)		Straw (kg DM/d)		Proportion of concentrate	
Diet:	0-14d	15-28d	0-14d	15-28d	0-14d	15-28d
C	4.37	4.72	0.90	0.52	82.7	89.4
T	3.89	4.70	0.78	0.51	83.2	90.1
M	3.90	4.97	0.69	0.44	84.2	91.8
Average	4.05	4.79	0.79	0.49	83.3	90.4
SEM	0.221	0.305	0.069	0.053	1.33	1.04
Effects (*P*- value)						
Diet	0.56		0.88		0.67	
Time	0.002		0.006		<0.001	
Diet x Time	0.32		0.83		0.98	

SEM: standard error of means.

584 **Table 3.** Main effects of diet and day of sampling on pH, total volatile fatty acids (VFA) concentration (mM), molar VFA proportions
585 (mmol/mmol), lactate (mM) and ammonia (mg/L) concentration in the rumen of beef calves given different diets (a non-supplemented diet
586 ,C; C plus 20 g/kg of a chestnut and quebracho tannin extract, T; and C plus 6 g/kg of a mixture of medium-chain fatty acids, M) during the
587 adaptation period to a high-concentrate feeding. d0 to d28 indicate days of sampling from the beginning of the trial.

	pH	VFA	Acetate	Propionate	Butyrate	Valerate	BCFA	Lactate	Ammonia
Diet									
C	6.51	87.9^{a}	0.573	0.207	0.160	0.016	0.044	0.65	82.5
T	6.66	73.8^{b}	0.582	0.191	0.167	0.018	0.042	0.54	81.6
M	6.54	77.1^{ab}	0.536	0.239	0.159	0.023	0.042	0.55	80.2
SEM	0.056	3.93	0.0157	0.0162	0.0116	0.0003	0.0053	0.066	9.08
Time									
d0	6.92^{a}	80.7^{b}	0.683^{a}	0.187	0.093^{b}	0.008^{c}	0.029^{c}	0.15	88.6
d7	6.63^{b}	70.0^{b}	0.529^{bc}	0.212	0.187^{a}	0.015^{b}	0.057^{a}	0.54	85.8
d14	6.57^{b}	73.8^{b}	0.541^{bc}	0.203	0.185^{a}	0.024^{a}	0.048^{ab}	0.75	95.8
d21	6.44^{cb}	72.5^{b}	0.494^{c}	0.247	0.187^{a}	0.025^{a}	0.047^{ab}	0.61	81.9
d28	6.30^{c}	100.8^{a}	0.572^{b}	0.214	0.158^{a}	0.024^{a}	0.032^{bc}	0.84	56.0
SEM	0.072	5.08	1.8438	0.0171	0.0142	0.0021	0.064	0.071	10.95
Effects (*P*-value)									
Diet	0.16	0.035	0.12	0.14	0.89	0.12	0.93	0.43	0.98
Time	<0.001	<0.001	<0.001	0.13	<0.001	<0.001	0.013	<0.001	0.11
Diet x Time	0.17	0.32	0.79	0.87	0.5	0.065	0.93	0.033	0.66

588 SEM: standard error of means. BCFA: branched-chain fatty acids. $^{a, b, c}$ Different letters within a column indicate statistical differences at $P<0.05$.

589 **Table 4**. Proportion of the main groups of protozoa (±SD) and number of harboring beef calves
590 (out of 18) given different diets (a non-supplemented diet ,C; C plus 20 g/kg of a chestnut and
591 quebracho tannin extract, T; and C plus 6 g/kg of a mixture of medium-chain fatty acids, M)
592 during the adaptation period to a high-concentrate feeding. .

Day	Isotricha	Dasytricha	Entodinium	Epidinium	Subfamily Diplodiniinae
0	0.050 (±0.030)	0.199 (±0.138)	0.537 (±0.203)	0.131 (±0.107)	0.182 (±0.106)
n	18	17	18	6	18
7	0.014 (±0.014)	0.005 (±0.003)	0.981 (±0.020)	0.015 (±0.009)	0.004 (±0.003)
n	12	9	16	4	7
14	0.009 (±0.008)	0.043 (±0.055)	0.955 (±0.048)	0.072 (±0.047)	0.023 (±0.019)
n	10	3	17	5	8
21	0.032 (±0.050)	0.061 (±0.075)	0.928 (±0.104)	0.113 (±0.036)	0.032 (±0.048)
n	10	2	18	5	9
28	0.086 (±0.072)	-	0.932 (±0.075)	0.066 (±0.056)	0.027(±0.046)
n	9	0	18	4	7

Table 5. Spearman's correlations *(r, P)* between concentrations of different protozoal groups and of rumen fermentation variables. Only correlations with $P < 0.01$ are shown.

	n	pH	Butyrate	Valerate	BCFA	Lactate	Ammonia
Total protozoa	87		0.25, 0.017		0.48, <0.001	0.38, <0.001	0.35, <0.001
Entodinium	87		0.30, 0.004		0.48, <0.001		0.32, 0.002
Subf. Diplodiniinae	50	0.54, <0.001	-0.35, 0.003	-0.62, <0.001		-0.39, <0.001	

No relationships were established between *Dasytricha* or *Epidinium* and any fermentation parameters because of the low number of data pairs (31 and 24, respectively).

598 **Figure 1.** Ruminal protozoa concentration in beef calves during the adaptation period to a high
599 concentrate ration without supplement (○) with 2 g/kg of tannin extract (■), or with 6 g/kg of
600 medium-chain fatty acids mixture (▲). Upper bars show the standard error of the means.

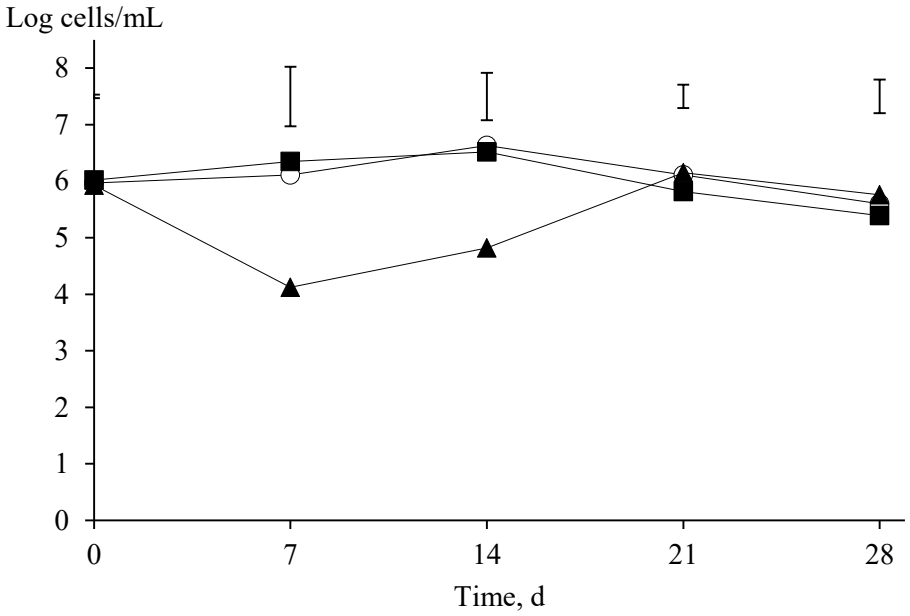

Figure 2. Ruminal concentration of protozoal groups (*Isotricha*, *Dasytricha*, *Entodinium*, *Epidinium* and Subfamily Diplodiniinae) in beef calves during the adaptation period to a high concentrate ration without supplement (○) with 2 g/kg of tannin extract (■), or with 6 g/kg of medium-chain fatty acids mixture (▲). a, b: Different letters indicate statistical differences at $P<0.05$. Upper bars show the standard error of the means.