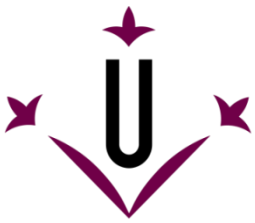

Effects of protein restriction on performance, ruminal fermentation and microbial community composition in Holstein bulls fed high-concentrate diets

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[1]**Abbreviations**: ADG, average daily gain; AIA, acid insoluble ash; BW, body weight; CP, crude protein; CTR, control; DM, dry matter; EE, ether extract; FIN, finishing; GRO, growing; LP, low protein; N, nitrogen; OM, organic matter; OTU, operational taxonomic unit; PLS-DA: partial least squares-discriminant analysis; VFA, volatile fatty acids.

ABSTRACT

The aim of this work was to study the impact of a crude protein (CP) restriction on performance, ruminal fermentation and microbial community composition in fattening Holstein bulls (from 118 to 331 d of age and from 165 to 522 kg body weight [BW]) fed high-concentrate diets. Twenty animals were assigned to two dietary treatments: concentrate CP was formulated either based on the levels used commercially (CTR: 140g CP/kg dry matter [DM]) or reducing them (LP: 120 g CP/kg DM). Concentrate was supplemented with barley straw and both were supplied *ad libitum*. Animal BW and concentrate intake were automatically recorded on a daily basis. Feces, urine and ruminal fluid were sampled twice, during the growing period (160 d of age and 225 kg BW) and during the finishing period (280 d of age and 444 kg BW), for digestibility, ruminal fermentation and microbial population characterization. No differences in BW or cumulated concentrate intake were found between treatments, despite the fact that average daily gain was lower in LP group at the beginning of the growing period ($P<0.001$). Crude protein limitation did not penalize dry matter ($P=0.654$) or organic matter ($P=0.526$) apparent digestibility, but it did affect CP apparent digestibility during the finishing period ($P=0.042$). Nitrogen (N) excretion was greater in CTR animals ($P=0.017$). Regardless of treatment ($P=0.511$), ruminal ammonia-N concentration was low (4.36±1.01 mg/L). Even though 135 OTUs (out of 489) were shared between treatments and periods (gathering 98.7% of analyzed sequences), ruminal microbial community composition was different between periods ($P=0.003$) and also between diets in either growing ($P<0.001$) or finishing ($P=0.046$) bulls. Bacteroidetes, Firmicutes and Actinobacteria were the three dominant phyla and *Prevotella ruminicola* was the most abundant species. Ruminal microbial biodiversity was low but increased with age ($P=0.002$ for Shannon index and $P=0.035$ for Simpson index), as well as, ruminal microbial heterogeneity. Crude protein limitation increased functional interdependency among microbial genera, so LP-fed bulls were found to

have a more complex microbiota community structure than CTR-fed bulls. No relevant correlations between microbial genera and ruminal fermentation parameters were detected.

KEY WORDS

Bulls; concentrate; microbiota; protein restriction; rumen.

1. INTRODUCTION

Ammonia produced by livestock is a main pollutant that contributes to eutrophication, soil acidity and aerosol formation, impairing atmospheric visibility and human health (Hristov et al., 2011). Moreover, animals and their waste also emit methane and nitrous oxide gases, which are contributors to global warming.

Ruminants are inefficient dietary nitrogen (N) utilizers and, in a common beef cattle fattening system, from 10 to 20% of N intake is retained, from 30 to 50% is excreted in feces and from 40 to 70% is excreted in urine (Cole and Todd, 2008). Available research data indicate that diet has strong effects on ammonia emissions from excreted manure and, in beef cattle, a minimum requirement of 150-160 g crude protein (CP)/ kg dry matter (DM) for growing Holstein bulls is generally accepted (NRC, 2000).

Lowering dietary CP content may compromise animal performance, which would be undesirable for most producers. However, this is not always the case. Using high-concentrate diets, a significant CP reduction from 170 g/kg to 140 g/kg (on a DM basis) did not alter the average daily gain (ADG) in crossbred heifers (Devant et al., 2000). Similar results were obtained when decreasing CP from 135 g/kg to 119 g/kg (on a DM basis) in Holstein heifers receiving 70/30 roughage to concentrate diets (Zhang et al., 2017). Going further, Erickson and Klopfenstein (2001) were able to reduce CP inputs by 10% in crossbred animals maintaining ADG. Therefore, young cattle fed high-concentrate diets seem to be able to adapt to a protein supply reduction, maintaining growth performance and reducing N waste. However, it remains unclear if such adaptation is accomplished throughout animal metabolism itself or throughout its ruminal symbiotic microbiota.

Ammonia is the main N source for microbes and a minimum level of ammonia-N concentration in the rumen (50 mg/L) has been defined to fulfil microbial N requirements (Satter and Slyter, 1974). However, several authors are critical with the definition of a constant

threshold level and microbial ammonia-N requirements may be dependent on fermentable organic matter (OM) availability (Song and Kennelly, 1990) and/or presence of preformed protein (Broudiscou and Jouany, 1995). Other authors outline a differential ammonia-N level to attain either the maximum microbial protein yield or DM degradation (Balcells et al., 1993). Intensive research has been done to describe the relationship between CP availability and microbial yield (Hoover and Stokes, 1991); however, much less research has been conducted to explore the impact of CP availability on rumen microbiota. Chanthakhoun et al. (2012) demonstrated an increase in total bacteria counts with CP availability, whereas Yang et al. (2016) could not confirm such findings.

Increasing CP supply raised proteolytic bacteria abundance (*Butyrivibrio fibrisolvens* and *Prevotella ruminicola*) in crossbred beef steers fed total mixed ration (Wang et al., 2017), though such effect was not that clear on other microbial populations: the abundance of cellulolytic bacteria (*Ruminococcus albus, Ruminoccocus flavefaciens* and *Fibrobacter succinogenes*) significantly increased with dietary CP supply (Wang et al., 2017), while other authors could not detect such shift (Yang et al., 2016). Protozoa and fungi counts remained unchanged with increasing levels of CP supply but archaea counts were found to be superior (Chanthakhoun et al., 2012).

Most of the existing studies are focused on titers and/or properties of singular microbes in response to specific challenges (i.e. dietary treatments), leaving microbiota interactions unexplored. Considering rumen ecosystem complexity, microbial interactions may explain relevant aspects of rumen functioning, so searching for new tools to reveal the impact of nutritional challenges on the whole ecosystem under real conditions is of great interest. In this sense, some studies of rumen microbiota across different ruminant species (Kittelmann et al., 2013) and diets (Kumar et al., 2015) have demonstrated that rumen microbiota profile and function are determined by factors still unexplored (Henderson et al., 2015).

Bearing in mind that the possibility of reducing N supply without impairing productive results could be feasible, this assay was built with a double objective: assess the impact of reducing dietary CP from 140 g/kg to 120 g/kg (on a DM basis) on animal performance and to analyze rumen microbiota adaptation to such shortage, in Holstein bulls raised under commercial intensive feeding system.

2. MATERIALS AND METHODS

2.1 Animals, diets and housing

This experiment was conducted at the research facilities of *Cooperativa d'Ivars d'Urgell, SCCP* (Ivars d'Urgell, Spain, 41°41'50"N, 0°58'53"E) between January and August 2017. All procedures were carried out under Project License CEEA 01-07/16 and approved by the in-house Ethics Committee for Animal Experiments at the University of Lleida. Care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purpose.

Twenty Holstein bulls (mean ± standard error: 118 ± 1 d of age) were group-housed in two outdoor paved and covered pens equipped with two feedbunks each: an individual one for concentrate and a common one for straw. According to body weight (BW), animals were assigned to two experimental treatments, consuming two corn-based concentrates and barley straw (*Table 1*). Experimental concentrates were formulated to reach two levels of CP: one with 140 g/kg (on a DM basis), which was considered as control (CTR) because it represented the conventional CP level used by beef cattle producers in the north-east region of Spain (*Cooperativa d'Ivars d'Urgell, SCCP*), and the other one with 120 g/kg (on a DM basis), which was considered as a low protein (LP) concentrate. Feedbunks were filled once daily at

08:00h and animals had free access to drinking water. One animal in the LP group was excluded from the trial due to a respiratory illness.

2.2 Measurements and sampling

The experimental period included the whole fattening phase. The first 150 d of the trial were considered to be the growing period, beginning when animals were 118 ± 1 d of age and 165 ± 3 kg of BW and ending when they were 268 ± 1 d of age and 435 ± 8 kg of BW. After that, the finishing period started and it continued until animals reached the commercial slaughter weight (331 ± 1 d of age and 522 ± 9 kg of BW).

Daily animal BW and concentrate consumption were automatically controlled by a feed station. Briefly, it was equipped with a feedbunk and an individual tunnel-type feeder both provided with a scale. When a calf entered the feeder, it was identified, its BW was registered and concentrate intake was obtained by difference between initial and final feedbunk weight. Both concentrate and straw, which was the only source of roughage, were supplied *ad libitum* in a free-choice system.

Feces, urine and ruminal fluid samples were taken on days 37, 38, 42 and 43 of the trial, corresponding to growing period (GRO) (160 ± 1 d of age and 225 ± 5 kg of BW), and on days 160, 161, 162 and 163 of the trial, corresponding to finishing period (FIN) (280 ± 1 d of age and 444 ± 9 kg of BW) for digestibility, ruminal fermentation and microbial population characterization.

Fecal excretion and straw intake were calculated based on concentrate intake and by a double marker system using chromium oxide (Cr_2O_3) as external marker and acid insoluble ash (AIA) as internal marker. Chromium oxide was mixed with concentrate (150 mg/kg in GRO and 90 mg/kg in FIN) and administered during 15 d. Fecal samples (approximately 50 g, one sample per animal and day, obtained between 9:00 – 13:00 a.m. during the four last days of chromium oxide administration) were collected using rectal stimulation and stored at -20ºC until marker

146 determination (both Cr_2O_3 and AIA) and proximate chemical analysis. After thawing, fecal
147 samples from each animal were pooled and mixed to produce one grab sample per collection
148 period. During sampling days, concentrate and straw samples were also collected and stored
149 at 5ºC until marker and proximate analysis.
150 Urine samples (10 mL, one sample per animal and collection period [GRO and FIN], obtained
151 between 9:00 – 13:00 a.m.) were taken by prepuce stimulation. Samples were strained to
152 remove hair and debris, immediately frozen in dry ice and stored at -80ºC until N and
153 creatinine analysis.
154 Ruminal fluid samples (one sample per animal and collection period [GRO and FIN], obtained
155 between 9:00 – 13:00 a.m.) were collected using an oral stomach tube connected to a vacuum
156 pump. Each sample was obtained through two sequential collections: firstly, ruminal fluid
157 (approximately 200 mL) was collected and discarded to avoid sample's contamination with
158 saliva that possibly got into the tube during its introduction through the animal's mouth and
159 esophagus. After that, ruminal fluid (approximately 200 mL) was collected again, strained
160 through a cheese-cloth and its pH recorded (Testo 205, Testo AG, Germany). Then, 15 mL of
161 ruminal fluid was immediately frozen with dry ice and stored at -80ºC for subsequent DNA
162 extraction and molecular analysis. The remaining ruminal fluid was sampled for ammonia-N
163 (2 mL over 0.8 mL of 0.5 N HCl) and volatile fatty acids (VFA) concentration (4 mL over 1
164 mL solution of 0.4 M ortho-phosphoric acid and 0.02 M 4-methylvaleric acid as internal
165 standard, in distilled water). Samples were immediately frozen with dry ice and stored at -
166 20ºC until analysis.

2.3 Chemical analysis

Feed and feces DM (index nº 934.01), ash (index nº 942.05) and ether extract (EE) (index nº 2003.05) contents were determined according to the AOAC methods (AOAC, 2006), as well as N content (index nº 990.03) in feed, feces and urine.

Neutral detergent fiber and acid detergent fiber analyses in feed were carried out following the sequential procedure of Van Soest et al. (1991), with the Ankom200/220 fiber analyzer (Ankom Technology, USA). Neutral-detergent fiber was assayed with a heat stable amylase.

Chromium in feed and feces was analyzed as follows. Samples (0.5 g) were calcined (550ºC, 2h) and digested with 3 mL HCl (1:1) in a sand bath (60ºC, until dry). The residue was then dissolved with 3 mL HCl (1:1), filtered and washed with 50 mL of hot distilled water. Chromium concentration was quantified by inductively coupled plasma mass spectrometry (7700x, Agilent Tecnologies, USA).

Acid insoluble ash was analyzed according to a standard procedure (BOE, 1995) based on the method of Shrivastava and Talapatra (1962). Briefly, the residue of ash content determination was introduced in an Erlenmeyer flask and then hydrolyzed with 75 mL of 3N HCl and boiled for 15 minutes. The sample was then filtered through ash-free filter paper (cat nº 1004 150, Whatman) and washed the residue with 50 mL of hot distilled water. The filter with the residue was dried (103ºC, 2 h) and then ashed (550ºC, 3 h) in a tared crucible. The crucible and its content were cooled in a desiccator at room temperature and weighed to calculate the AIA content.

Creatinine was determined using ultra high performance liquid chromatography coupled with mass spectrometry, using an adaptation of Boudra et al. (2012). The chromatographic system was an Acquity module (Waters Corporation, USA) and peak separation was performed with an Acquity UPLC® BEH Amide 1.7 µm column (150*2.1 mm, Waters Corporation, USA) using a gradient solvent system (solvent A: acetonitrile with 1 mL/L formic acid; solvent B:

192 methanol-water solution 1:49 with 1 mL/L formic acid). The gradient conditions were as
193 follows: the initial percentage of solvent B was 20%, which was raised to 40% between
194 minutes 1 and 2 and then kept constant for 2 more minutes. After that, it was lowered to 20%
195 in 0.01 minutes and finally kept constant for the last 1.49 minutes of the analyses. The initial
196 flow rate was 0.4 mL/min, which was kept constant for 2 minutes, then raised to 0.5 mL/min
197 in 0.1 minutes and kept constant for 1.90 minutes. After that, it was lowered to 0.4 mL/min in
198 0.01 minutes and kept constant for the last 1.49 minutes of the analysis. The injection volume
199 was 5 µL and the auto sampler and column temperature were maintained at 10ºC and 30ºC
200 respectively. Finally, mass spectrometric analyses were performed on an Acquity TQD triple
201 quadrupole tandem mass spectrometer (Waters Corporation, USA).
202 Ammonia-N concentration was determined by the Chaney and Marbach (1962) method after
203 sample centrifugation (13,800 g, 30 min).
204 Volatile fatty acids concentration and molar VFA profile were determined by gas
205 chromatography according to the technique proposed by Jouany (1982), using a capillary
206 column (GS-BR-SWAX 30m x 0.25 mm D.I. x 0.25 µm, Bruker, USA).
207 **2.4 DNA extraction and sequencing**
208 DNA extraction was carried out on freeze-dried ruminal fluid (the initial sample amount was
209 60 mg) through physical disruption (1 min) using a bead beater (Mini-bead beater 1, BioSpec
210 Products, USA) and subsequent DNA purification with the QIAamp DNA Stool Mini Kit (ID:
211 51504; QIAGEN N.V., Germany), with the modifications of greater temperature (95ºC) to
212 improve cell lysis and greater elution time (3 min). DNA was amplified by using the following
213 primer set:
214 Forward = 5':
215 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse = 5':

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC

C

which target the V3 and V4 regions of the microbial 16S rRNA. Sequencing was conducted on an Illumina MiSeq 2x300 platform. Sequencing of 16S RNA genes was performed by Era7 Bioinformatics (Spain).

2.5 Bioinformatics

Sample reads were assembled by using FLASh software (Fast Length Adjustment of Short reads (Magoč and Salzberg, 2011) and quality filtered using FastQC software (Babraham Bioinformatics, Babraham Institute, U.K.). Operational Taxonomic Units (OTUs) were picked using BLAST software (Basic Local Alignment Search Tool, National Center for Biotechnology Information [NCBI], USA) with a 98% similarity threshold. Taxonomy assignment of OTUs was performed by comparing sequences to the *Taxonomy* database by NCBI (USA), according to the Lowest Common Ancestor method.

2.6 Calculations and statistical analysis

Fecal excretion was calculated using chromium concentration in concentrate and feces, as follows (Eq. 1):

$$(Eq.1)\ Fecal\ excretion\ = \frac{[Cr]c * CI}{[Cr]f}$$

Where *[Cr]c* and *[Cr]f* are chromium concentration in concentrate and in feces and *CI* is concentrate intake.

Straw intake was calculated using estimated fecal excretion (Eq. 1) and AIA concentration in both feed and feces, as follows (Eq. 2):

$$(Eq.2)\ Straw\ intake = \frac{[AIA]f * FE - [AIA]c * CI}{[AIA]s}$$

239 Where *[AIA]f*, *[AIA]c* and *[AIA]s* are AIA concentration in feces, concentrate and straw,
240 respectively, *FE* is fecal excretion and *CI* is concentrate intake.
241 Total tract apparent digestibility of nutrients was calculated using concentrate intake,
242 estimated straw intake (Eq. 2), estimated fecal excretion (Eq. 1) and nutrient concentration in
243 both feed and feces, as follows (Eq. 3):

244 $$(Eq.3)\ Apparent\ digestibility\ coefficient\ = \frac{([N]c * CI + [N]s * SI) - ([N]f * FE)}{([N]c * CI + [N]s * SI)}$$

245 Where *[N]c, [N]s, [N]f* are nutrient concentration in concentrate, straw and feces, respectively,
246 *CI* and *SI* are concentrate and straw intake, respectively and *FE* is fecal excretion.
247 Urine daily volume was determined assuming a creatinine constant urinary output of 883 μmol
248 per kg of metabolic weight and day (Chen et al., 1992).
249 Performance, intake, apparent digestibility, ruminal fermentation parameters, N balance,
250 microbial abundance and microbial biodiversity data were analyzed with a mixed model;
251 including treatment (CTR *vs* LP), period (GRO *vs* FIN) and their interaction as fixed effects
252 and animal as random effect, to account for repeated measurements (R Core Team, 2018; lme4
253 package). Comparisons among groups were performed by the Tukey's method (R Core Team,
254 2018; emmeans package); additionally, only for microbial taxa abundance, false discovery
255 rate was addressed using Benjamini-Hochberg statistical test. Individual sample within three
256 standard deviations of the mean were discarded and not included to the statistical analysis.
257 Significant effects were declared at $P<0.05$ and tendency to difference at P between 0.05 and
258 0.10.
259 Sequence data were normalized and biodiversity indexes were calculated (R Core Team, 2018;
260 Vegan package). To determine the proportion of shared and unshared OTUs, a Venn diagram
261 was performed (R Core Team, 2018; VennDiagram package). To determine the impact of both
262 treatment and period on the microbial community structure, a permutational multivariate
263 analysis of variance (Adonis) was conducted based on the Bray-Curtis dissimilarity index and

calculating statistical significance after 5,000 random permutations (R Core Team, 2018; Vegan package). A partial least squares-discriminant analysis (PLS-DA) on community structure data was performed for graphical interpretation (R Core Team, 2018; mixOmics package). Correlation analyses were performed to decipher the interactions within rumen microbial community as well as between rumen microbial community and fermentation patterns. Spearman correlation analysis was performed between all microbial genera and between all microbial genera and rumen fermentation parameters (pH, ammonia-N, VFA, acetate, propionate and butyrate concentrations). Only those genera present in more than 50% of the individuals and only correlations coefficients larger than 0.6 and P-values (adjusted in the case of within genera correlations) below 0.05 were further included in the correlation network. Microbial correlation network was generated using igraph package (R Core Team, 2018). Microbial network complexity was described in terms of number of nodes (genera), number of edges (positive or negative correlations) and betweenness (measure of centrality in a graph based on shortest paths).

3. RESULTS

3.1 Animal's performance through the whole experimental period

Data on animal performance evolution through the whole fattening process is showed in *Fig. 1*: animals began with 158 (± 2) kg of BW and they finished when they reached the commercial slaughter weight (511 ± 5 kg). During the first month of the experimental period, CTR animals presented a higher ADG than LP ones (*Fig. 1*A; $P<0.001$); however, live weight tended to equilibrate throughout the experimental time-course (only at day 90 CTR animals tended to weigh more than LP animals; *Fig. 1*A; $P=0.075$). No differences in cumulated concentrate intake were found (*Fig. 1*B).

3.2 Intake, apparent digestibility and N balance

In *Table 2*, data on DM intake (both concentrate and forage) are presented. Animals increased voluntary intake with age although such increase was more pronounced in LP animals than in CTR ones. Increased DM intake was mainly explained by concentrate intake (from 5.29 to 8.17 kg) although straw ingestion also incremented significantly (from 0.77 to 1.37 kg).

Data on DM, OM and CP apparent digestibility are shown in *Table 2*. No differences in DM or OM apparent digestibility were detected, either between treatments or between periods. Crude protein apparent digestibility was impaired in LP-fed animals during the finishing period, but not in the growing.

Data on N balance is also presented in *Table 2*. As expected, N intake and N excretion were higher in CTR-fed bulls than in LP-fed ones.

3.3 Ruminal fermentation

Data on ruminal fermentation are presented in *Table 3*. Ruminal pH decreased with period and ammonia-N concentration was higher in finishing than in growing bulls. No differences were detected between experimental diets although numerical variations in mean values registered in the growing period were consistent; in this sense, it is necessary to remark the notorious residual variation observed in ammonia-N concentrations (C.V.=143%).

Ruminal VFA concentration increased with bulls age and it was higher in CTR animals than in LP ones. Acetate proportion did not vary among treatments. Comparing to the rest of the treatments, LP-fed bulls presented a lower propionate proportion, which led to a significant treatment-by-period interaction. Butyrate proportion was higher in CTR-fed animals than in LP ones in the growing period but not in the finishing one.

3.4 Ruminal microbiota profile

3.4.1 Data set features

After sequencing and normalization procedures, an average of 22 658 sequences per sample were obtained, resulting in 860 996 sequences from the 38 samples. A total 489 OTUs were obtained at the 98% sequence similarity cut-off levels with 114 ± 5 (range: 56 to 169) as the mean number of OTUs per sample. Good's coverage value was 99.8% suggesting that more than 99% bacterial and archaeal phylotypes were identified. Unclassified mean rates of OTUs at family and genus level were 0.52% (0.06-4.08%) and 0.81% (0.15-5.18%), respectively. Operational taxonomic units were assigned to 2 kingdoms, 12 phyla, 24 classes, 45 orders, 92 families and 214 genera. Shared taxa by all individuals in each treatment and sampling period were deemed to be core bacterial/archaeal communities. Out of a total number of 489 OTUs, 135 (27.6%) were shared by all experimental groups (*Fig. 2*A) and were unchanged across treatments and periods. At a sequence level, the proportion of sequences belonging to the common OTUs was as high as 98.7%. Composition of the core bacterial and archaeal community is exhibited in *Fig. 2*B and almost 80% of these taxa belonged to *Prevotella* genus.

3.4.2 Microbial community structure and diversity

Phyla and genera abundance are presented in *Table 4.* Bacteroidetes and Firmicutes were the predominant bacterial phyla in ruminal fluid. Bacteroidetes abundance decreased in the finishing period only in CTR-fed animals; while Firmicutes abundance followed the opposite pattern. Actinobacteria, Euryarchaeota, Proteobacteria and Fibrobacteres abundance were not affected either by treatment or period.

Prevotella, the most abundant genus in ruminal fluid, decreased with age in CTR-fed animals but not in LP ones. Differences in *Roseburia* and *Olsenella*, which were more prevalent in finishing than in growing animals, were not related to treatment. *Selenomonas* was more abundant in GRO than in FIN. *Bifidobacterium* titers were clearly greater in GRO than in FIN,

335 especially for LP-fed. *Agathobacter* presence rose as animals aged, in a more intense manner
336 in CTR-fed animals. *Sharpea* was richer in finishing animals fed CTR diets: those differences
337 could be explained both by treatment and period effects. *Ruminococcus* was more relevant in
338 older animals but it was not altered by treatment. Presence of *Pseudobutyrivibrio* was found
339 to be independent of diet and bulls age.
340 Shannon and Simpson biodiversity indexes data are also presented in *Table 4*: their values
341 were similar across treatments and sampling periods except from growing bulls fed CTR diets,
342 which exhibited significantly lower values. Richness values were proved to be independent of
343 the experimental conditions.
344 Adonis test indicated that differences in bacterial communities were significant between
345 periods ($P = 0.003$) and also between diets in either GRO ($P<0.001$) and FIN ($P=0.046$), as it
346 can be deduced by the graphical representation of PLS-DA (*Fig. 3*).
347 3.4.3 Microbial network and interactions with ruminal parameters
348 Microbial networks were performed to test how bacteria and archaea interact between them
349 (*Fig. 4* and *Fig. 5*). Relating to the degree of interaction, we studied the number of taxa (nodes)
350 that establish significant interactions (edges) with other taxa. In GRO, animals fed a CTR diet,
351 in comparison to a LP diet, had a lower microbial network complexity in terms of nodes (11
352 in CTR vs 26 in LP) and edges (7 in CTR vs 25 in LP). These differences became even more
353 pronounced in FIN, again in terms of nodes (9 in CTR vs 22 in LP) and edges (8 in CTR vs
354 40 in LP). Thereafter, we investigated microbial taxa that act as main information gateways in
355 networks. Control networks had no nodes lying on paths that connected other nodes, so
356 betweenness centrality values were 0. Contrarily, LP networks had mean betweenness
357 centrality values of 0.23 in GRO and 1.82 in FIN. The genus with the highest centrality value
358 in GRO was *Pseudomonas,* lying in 6 interconnecting paths; in FIN, the most central genus
359 was *Microbacterium,* being part of 23 interconnecting paths.

Correlations between genera abundance and fermentation parameters (*Supplementary Table 1* and *Supplementary Table 2*) varied greatly with period in the case of LP animals: in GRO, 26 genera were found to establish 60 significant correlations with fermentation parameters studied; while in FIN, only 16 genera established 12 significant correlations. Contrarily, in CTR animals, the number of significant correlations and genera involved remained stable, regardless of period (32 genera involved in 20 significant correlations in GRO and 28 genera involved in 20 significant correlations in FIN). When comparing CTR and LP-fed animals, only the abundance of few genera showed consistent correlations with fermentation parameters under both dietary treatments in at least one period: *Prevotella* positively correlated with propionate and some Firmicutes (*Pseudobutyrivibrio, Roseburia, Ruminococcus)* showed positive correlations with acetate and negative with propionate. However, most correlations between microbes and ruminal fermentation parameters were detected under only one dietary treatment and period.

4. DISCUSSION

4.1 Performance, intake, digestibility, ruminal fermentation and nitrogen balance

Feeding low CP diets optimizes diet cost and reduces N waste. In relation to the latter, animals fed low CP diet excreted less N than those fed CTR diets (1040 mg/kg $BW^{0.75}$ for CTR animals vs 850 mg/kg BW^{075} for LP animals). However, the question that remains is: does such challenge compromise bulls' performance? It is true that, initially (118-163 d of age), a low but significant reduction in ADG was observed in LP-fed animals; however, this group recovered its growth rate or grew even faster than CTR-fed animals. The reduced growth in young animals caused by a dietary protein restriction has already been reported in the existing literature (Segers et al., 2014), as well as the lack of effect of protein restriction on finishing animal performance (Dong et al., 2017).

No relevant discrepancies were stated between our reported ADG or DM intake and that from available literature (Verdú et al., 2017), in intensively reared Holstein bulls consuming corn-based concentrate and straw *ad libitum* in a free choice system..

Nutrient apparent digestibility rates were similar to those reported elsewhere (Devant et al., 2000) with animals fed high-concentrate diets. Crude protein was the only nutrient whose apparent digestibility was negatively affected by dietary CP limitation, as the consulted literature has already described, either in growing (Devant et al., 2000) or in finishing animals (He et al., 2018).

In theory, animals receiving high-concentrate diets would have an intense VFA production that would induce a critically low ruminal pH, leading to subacute or acute ruminal acidosis. However, that was not the case, and recorded ruminal pH values in the present study were in agreement with those proposed by other authors working in similar conditions (Rotger et al., 2006); suggesting that young ruminants may be able to adapt to specific challenges, such as high-concentrate diets. Hypothetically, the young rumen seems to be malleable enough to sustain extreme fermentation conditions and the role of ruminal microbial community in such process may be crucial.

In ruminants, ammonia-N concentration in rumen is commonly used to check the adequacy of N supply for microbial growth. On average, ruminal ammonia-N concentration was very low, especially in growing bulls fed the LP diet. The optimal ruminal ammonia-N concentration for maximal microbial growth has been fixed at 50 mg/L (Satter and Slyter, 1974). However, the existence of a threshold level for maximal microbial growth has long been questioned because N requirements may vary in function of ruminal fermentation conditions (Balcells, 1990). It has been recognized that the necessary ammonia-N concentration to fulfill microbial requirements is lower when high-concentrate diets are employed (Ludden and Cecava, 1995; Devant et al., 2000, 2001) and, in such situation, microbes may preferentially utilize

410 alternative N sources such as soluble amino acids or preformed peptides (Williams and
411 Cockburn, 1991). In a similar sense, Russell et al. (1983) showed that non-structural
412 carbohydrates fermenting bacteria derived 66% of their N from peptides or amino acids and
413 34% of their N from ammonia; so specific N incorporation mechanisms could allow the
414 surveillance and growth of certain microbial populations under low ammonia-N concentration.
415 Recorded ruminal VFA concentration was similar to those described in other trials employing
416 high-concentrate diets with rapidly degradable carbohydrates (Ludden and Cecava, 1995;
417 Devant et al., 2000); and it was only penalized by protein restriction in finishing animals, in
418 accordance with other authors working with animals of similar age (Ceconi et al., 2015).
419 Protein reduction also decreased propionate concentration in the growing period (Reynal and
420 Broderick, 2005), possibly due to the considerable quantities of propionate derived from the
421 degraded protein (Van Bruchen et al., 1985).

422 **4.2 Ruminal microbial community composition**

423 Rumen microbiota represents a complex network where bacteria, archaea, protozoa and
424 anaerobic fungi work in harmony contributing to the health and productivity of ruminants, as
425 described by Kumar et al. (2015). Factors that influence the composition of gut microbiota, as
426 well as other microbial communities, can include stochastic processes, such as dispersal,
427 genetic diversification and ecological drift (Pereira and Berry, 2017). However, deterministic
428 interactions between species, individuals and the environment also create defined niches and
429 thus might influence community composition. We hypothesized that rumen microbiome
430 changes, not only as ruminant animal ages, but under different dietary conditions and that
431 these microbial shifts may be responsible for resilience to protein reduction. For this reason,
432 we characterized ruminal bacterial and archaeal populations. Protozoa abundance in animals
433 fed high-concentrate diets is proved to be minimal due to the reduction of rumen pH caused
434 by this type of diets (Mackie et al., 1978); likewise, fungal population is known to be scarce

435 in diets rich in starch and soluble carbohydrates (Grenet et al., 1989), so neither protozoa nor
436 fungi were quantified in this study.
437 Bacteroidetes and Firmicutes were detected as the dominant phyla in rumen; Actinobacteria
438 was the third phylum in order of importance and Proteobacteria and Fibrobacteres were
439 scarcely found. According to existing studies of ruminal microbiota shifts when transitioning
440 to a high-concentrate diet (Fernando et al., 2010; Petri et al., 2013; Tapio et al., 2017),
441 Bacteroidetes abundance (aforementioned studies, 25-40%) does normally rise with increasing
442 amount of concentrate in diet (mainly due to a rise in its main component *Prevotella*).
443 Moreover, Bacteroidetes titers have been proved to be higher when (i) the liquid fraction of
444 ruminal content is analyzed, (ii) when the bead-beating time is short or (iii) when the QIAamp
445 DNA Stool Mini Kit is used as the DNA extraction method (Henderson et al., 2013), being all
446 those three conditions met in the present study. Actinobacteria role in ruminal fermentation is
447 not very well known (Šuľák et al., 2012) and, in our study, its abundance was higher than that
448 previously observed in animals in high-concentrate diet conditions (aforementioned studies,
449 0.5-1.5%). Proteobacteria abundance was low in comparison with the aforementioned studies
450 (13-20%) and a similar decrease has been previously related to feed restriction in bulls
451 (McCabe et al., 2015). Fibrobacteres minimal abundance was in accordance with the cited
452 literature in animals with such low fiber ingestion. High-concentrate diets are also known to
453 reduce archaeal abundance (Belanche et al., 2012; Tapio et al., 2017), so its minor presence in
454 the current study was already expected.
455 Microbial biodiversity levels in terms of richness, Shannon index or Simpson index were low
456 because they do normally experiment a decrease when animals are fed a high-concentrate diet
457 (Belanche et al., 2012; Petri et al., 2013; Tapio et al., 2017). The mentioned authors reported
458 higher biodiversity levels than ours (Richness: 138-148 OTUs/animal; Shannon index: 3.2-
459 4.6; Simpson index: 0.8-0.9) possibly because their studies used animals previously fed with

high-forage diets and, consequently, the initial microbial biodiversity in rumen could have been more elevated than that registered in our animals. Moreover, excluding Petri et al. (2013) trial, diets employed in the other studies did not reach the 10/90 forage-to-concentrate ratio.

In the transit from growing to finishing period, microbial biodiversity levels tended to evolve towards a more heterogeneous microbiota, which partially agrees with Jami et al. (2013) and Dill-McFarland et al. (2019) who reported an age-dependent increase in bacterial diversity, as well as in within-age-group similarity. More specifically, CTR animals presented lower alpha diversity values than the rest of animals and periods; leading us to think that, at this early period, CP level was limiting and promoted an increase in ruminal microbial biodiversity levels in order to overcome such substrate limitation (Langenheder and Prosser, 2008). Shabat et al. (2016) have outlined a possible relationship between performance and alpha diversity and it seems that more efficient animals present lower diversity and higher dominance of specific species. In this sense, the increased microbial biodiversity caused by the restriction in CP availability would have a detrimental effect on the efficiency of utilization of that nutrient by LP animals, which could ultimately be the reason of their reduced growth rate at the beginning of the fattening period.

Bulls' age did affect ruminal microbiota at phylum level: we found descending titers of Bacteroidetes and increasing titers of Firmicutes, while archaeal abundance was not altered by age in accordance with Liu et al. (2017a).

Rumen maturation did alter rumen microbiota also at genus level. *Prevotella* and *Bifidobacterium* abundance decreased while some Clostridiales (*Roseburia, Agathobacter, Ruminococcus)* and *Olsenella* increased. *Prevotella* genus can fill a variety of niches thanks to its diverse metabolic capabilities (it is mainly known for their starch-degrading and proteolytic capacity but they play a role in fiber degradation), being hard to interpret its ecological role in rumen (Stevenson and Weimer, 2007); its age-dependent decrease has

485 already been reported elsewhere (Liu et al., 2017a). *Bifidobacterium* genus is normally
486 abundant in the gastrointestinal tract of young mammals because milk glycans enhance its
487 proliferation (Pacheco et al., 2015); we hypothesize that, after weaning, *Bifidobacterium*
488 population started to progressively decrease its abundance in bulls' ruminal ecosystem, still
489 being detectable at low abundance in GRO but not in FIN. *Roseburia* and *Agathobacter* genera
490 are members of Clostridium Cluster XIV and they produce butyrate as a fermentation end
491 product (Rosero et al., 2016); to our knowledge, their age-dependent variation in rumen has
492 not been reported yet. *Ruminococcus* genus, which also increased its abundance as animals
493 aged in the study of Liu et al. (2017a), includes cellulolytic and non-cellulolytic species (La
494 Reau et al., 2016). *Olsenella* is a markedly peptidolytic and lactic acid producing genus that
495 is usually found in the gastrointestinal tract of both ruminants and non-ruminants (Kraatz et
496 al., 2011); its age-shift is not mentioned in any of the consulted studies.
497 In the present study and as animals matured, microbial co-occurrence pattern evolved to a
498 tighter and rich network but only in LP-fed individuals, while in CTR-fed ones microbial
499 network remained stable. The observed evolution in LP-fed animals agrees with similar
500 challenge in dairy cows: available literature reveals that the extent of co-occurrence among
501 microbial domains in young ruminants is naïve but, with rumen maturation, there is a greater
502 interdependency across microbial domains (Kumar et al., 2015).
503 Current data analyzing how the dominant ruminal microbiota species or their relationships
504 respond to a reduced protein intake is not conclusive (Wang et al., 2017; He et al., 2018).
505 However, in goats fed mixed or forage diets, the richness of fiber-, protein- and fat-degrading
506 bacteria increased with their specific substrate content in the ration (Liu et al., 2017b). In a
507 similar approach, Belanche et al. (2012) described that protein restriction could alter microbial
508 populations involved in carbohydrate degradation, having cellulolytic microbiota more
509 sensitivity to the challenge.

Animals fed LP diet presented higher levels of microbial biodiversity than CTR animals during the growing period, which to some extent agrees with data reported by Peng et al. (2017) who found increased bacterial diversity in pig's hindgut compartment when CP content was moderately reduced.

At phylum level, CP restriction reduced Firmicutes abundance, confirming previous values described by Luo et al. (2015) in the cecum and Fan et al. (2017) in the ileum of pigs.

At genus level, the CP optimization led to a lower abundance of the lactate producer *Sharpea* (Morita et al., 2008), which has not been seen by any of the aforementioned authors. In contrast, *Bifidobacterium* levels remained higher when dietary protein was reduced. Considering that Peng et al. (2017) observed just the opposite in their study, we suspect that the rise in *Bifidobacterium* titers might be due to the difference in EE content of the two dietary treatments (72 g EE/kg DM in LP concentrate and 51 g EE/kg DM in CTR concentrate), as this genus has been proved to be actively involved in lipid metabolism in rumen (Gorissen et al., 2010).

Considering that i) the proportion of shared sequences was extremely high, ii) unique OTUs gathered scarce sequences and iii) there were no major differences in alpha biodiversity levels between treatments, we expected that microbial network architecture under different dietary conditions would have been similar. However, LP-fed animals had a more complex ruminal microbial community in terms of microbial co-occurrence patterns than CTR-fed animals. The fact that microbial associative patterns are diet specific has already been observed in dairy cows fed diets with different forage-to-concentrate ratios (Kumar et al., 2015; Tapio et al., 2017). In the same line, an increasing degree of functional interdependence among genera with a progressive decrease in nutritional status has been described when comparing gut microbiome of healthy and malnourished children (Ghosh et al., 2014), so describing a similar phenomenon as that observed in the present study.

Functional redundancy, or the co-existence of functionally-similar organisms, is often considered to be an important feature of the gut ecosystem that contributes to robustness and resilience (Moya and Ferrer, 2016). However, some key metabolic activities may be restricted to one or few species, called "keystone" species or taxa. A keystone species has a large impact on the rest of the community and, in most occasions, has a disproportionally low abundance relative to its impact on the ecosystem (Mills et al., 1993). The fact that the vast majority of correlating genera were low-abundant supports the earlier assumption that minor species may hold key functions in rumen, for example, in the fermentation of proteins, mucins and toxic plant metabolites (Prins and Stewart, 1997; Tapio et al., 2017).

In our study we observed a general lack of agreement between the abundance of bacterial and archaeal genera and rumen fermentation parameters across diets. This suggests that functional diversity may occur even with similar taxonomical distribution (Belanche et al., 2019), relying on ruminal microbial community redundancy; i.e., the overlapping distribution of physiological capabilities across multiple microbial taxa (Weimer, 2015). For long, it has been known that the peripheral pathways of polymer cleavage are more diverse than the ensuing monomers processing pathways; so it seems that for every microbial function there are several candidates, no one identical to each other (Prins and Stewart, 1997).

5. CONCLUSIONS

Reducing CP content from 140 g/kg to 120 g/kg (on a DM basis) did not have major impacts on either DM intake or animal performance. Crude protein limitation did not penalize DM or OM apparent digestibility but it did reduce CP apparent digestibility and N waste. Statistical differences in ammonia-N due to dietary CP content could not be found. Low protein-fed bulls showed more diverse and complex ruminal microbiota with greater functional interdependency among genera; thus indicating that ruminal microbiota may be playing a crucial role in cattle resilience to protein restriction.

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LITERATURE CITED

AOAC, 2006. Official methods of analysis (18th ed). Association of Official Analytical Chemists, Gaithersburgs, MD, USA.

Balcells, J., 1990. La excreción urinaria de derivados de las purinas en el ganado ovino como índice del aporte de proteína microbiana al duodeno (Doctoral dissertation). University of Zaragoza, Zaragoza, Spain.

Balcells, J., Guada, J.A., Castrillo, C., Gasa, J., 1993. Rumen digestion and urinary excretion of purine derivatives in response to urea supplementation of sodium-treated straw fed to sheep. Br. J. Nutr. 69, 721–32.

Belanche, A., Doreau, M., Edwards, J.E., Moorby, J.M., Pinloche, E., Newbold, C.J., 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. J. Nutr. 142, 1684–1692. https://doi.org/10.3945/jn.112.159574

Belanche, A., Kingston-Smith, A.H., Griffith, G.W., Newbold, C.J., 2019. A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. Front. Microbiol. 10, 122. https://doi.org/10.3389/fmicb.2019.00122

BOE., 1995. Determinación de las cenizas insolubles en ácido clorhídrico. In: Real Decreto 2257/1994, de 25 de noviembre, por el que se aprueba los métodos oficiales de análisis

585 de piensos o alimentos para animales y sus primeras materias. BOE nº52, de 2 de marzo,
586 pp. 7161–7237.
587 Boudra, H., Doreau, M., Noziere, P., Pujos-Guillot, E., Morgavi, D.P., 2012.Simultaneous
588 analysis of the main markers of nitrogen status in dairy cow's urine using hydrophilic
589 interaction chromatography and tandem mass spectrometry detection. J. Chromatogr. B
590 Biomed. Sci. Appl. 1256, 169–176. https://doi.org/10.1016/j.chroma.2012.07.094
591 Broudiscou, L., Jouany, J.P., 1995. Reassessing the manipulation of protein synthesis by
592 rumen microbes. Reprod. Nutr. Dev. 35, 517–535. https://doi.org/10.1016/0926-
593 5287(96)80218-8
594 Ceconi, I., Ruiz-Moreno, M.J., DiLorenzo, N., DiCostanzo, A., Crawford, G.I., 2015. Effect
595 of urea inclusion in diets containing corn dried distillers grains on feedlot cattle
596 performance, carcass characteristics, ruminal fermentation, total tract digestibility, and
597 purine derivatives-to-creatinine index. J. Anim. Sci. 93, 357–369.
598 https://doi.org/10.2527/jas.2014-8214
599 Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and
600 ammonia. Clin. Chem. 8, 130–132. https://doi.org/10.1021/AC60252A045
601 Chanthakhoun, V., Wanapat, M., Berg, J., 2012. Level of crude protein in concentrate
602 supplements influenced rumen characteristics, microbial protein synthesis and
603 digestibility in swamp buffaloes (*Bubalus bubalis*). Livest. Sci. 144, 197–204.
604 https://doi.org/10.1016/J.LIVSCI.2011.11.011
605 Chen, X.B., Grubic, G., Ørskov, E.R., Osuji, P., 1992. Effect of feeding frequency on diurnal
606 variation in plasma and urinary purine derivatives in steers. Anim. Prod. 55, 185–191.
607 https://doi.org/10.1017/S0003356100037442

608 Cole, N.A., Todd, R.W., 2008. Opportunities to enhance performance and efficiency through
609 nutrient synchrony in concentrate-fed ruminants1,2,3. J. Anim. Sci. 86, E318–E333.
610 https://doi.org/10.2527/jas.2007-0444
611 Devant, M., Ferret, A., Calsamiglia, S., Casals, R., Gasa, J., 2001. Effect of nitrogen source in
612 high-concentrate, low-protein beef cattle diets on microbial fermentation studied in vivo
613 and in vitro. J. Anim. Sci. 79, 1944–1953. https://doi.org/10.2527/2001.7971944x
614 Devant, M., Ferret, A., Gasa, J., Calsamiglia, S., Casals, R., 2000. Effects of protein
615 concentration and degradability on performance, ruminal fermentation, and nitrogen
616 metabolism in rapidly growing heifers fed high-concentrate diets from 100 to 230 kg
617 body weight. J. Anim. Sci. 78, 1667–1676. https://doi.org/10.2527/2000.7861667x
618 Dill-McFarland, K.A., Weimer, P.J., Breaker, J.D., Suen, G., 2019. Diet influences early
619 microbiota development in dairy calves without long-term impacts on milk production.
620 Appl. Environ. Microbiol.85, e02141-18. https://doi.org/10.1128/AEM.02141-18
621 Dong, L.F., Zhang, W.B., Zhang, N.F., Tu, Y., Diao, Q.Y., 2017. Feeding different dietary
622 protein to energy ratios to Holstein heifers: effects on growth performance, blood
623 metabolites and rumen fermentation parameters. J. Anim. Physiol. Anim. Nutr. (Berl).
624 101, 30–37. https://doi.org/10.1111/jpn.12493
625 Erickson, G.E., Klopfenstein, T.J., 2001. Managing N inputs and the effect on N losses
626 following excretion in open-dirt feedlots in Nebraska. Sci. World J. 1 Suppl 2, 830–835.
627 https://doi.org/10.1100/tsw.2001.363
628 Fan, P., Liu, P., Song, P., Chen, X., Ma, X., 2017. Moderate dietary protein restriction alters
629 the composition of gut microbiota and improves ileal barrier function in adult pig model.
630 Sci. Rep. 7, 43412. https://doi.org/10.1038/srep43412
631 Fernando, S.C., Purvis, H.T., Najar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja, T.G.,
632 Roe, B.A., De Silva, U., 2010. Rumen microbial population dynamics during adaptation

633 to a high-grain diet. Appl. Environ. Microbiol. 76, 7482–7490.
634 https://doi.org/10.1128/AEM.00388-10
635 Ghosh, T.S., Sen Gupta, S., Bhattacharya, T., Yadav, D., Barik, A., Chowdhury, A., Das, B.,
636 Mande, S.S., Nair, G.B., 2014. Gut microbiomes of Indian children of varying
637 nutritional status. PLoS One 9, e95547. https://doi.org/10.1371/journal.pone.0095547
638 Gorissen, L., Raes, K., Weckx, S., Dannenberger, D., Leroy, F., De Vuyst, L., De Smet, S.,
639 2010. Production of conjugated linoleic acid and conjugated linolenic acid isomers by
640 *Bifidobacterium* species. Appl. Microbiol. Biotechnol. 87, 2257–2266.
641 https://doi.org/10.1007/s00253-010-2713-1
642 Grenet, E., Breton, A., Barry, P., Fonty, G., 1989. Rumen anaerobic fungi and plant substrate
643 colonization as affected by diet composition. Anim. Feed Sci. Technol. 26, 55–70.
644 https://doi.org/10.1016/0377-8401(89)90006-0
645 He, Y., Yu, Z., Qiu, Q., Shao, T., Niu, W., Xia, C., Wang, H., Su, H., Cao, B., 2018. Effects
646 of dietary protein levels and calcium salts of long-chain fatty acids on nitrogen
647 mobilization, rumen microbiota and plasma fatty acid composition in Holstein bulls.
648 Anim. Feed Sci. Technol. 246, 1–10. https://doi.org/10.1016/j.anifeedsci.2018.09.019
649 Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Collaborators, G.R.C., Janssen,
650 P.H., 2015. Rumen microbial community composition varies with diet and host, but a
651 core microbiome is found across a wide geographical range. Sci. Rep. 5, srep14567.
652 https://doi.org/10.1038/srep14567
653 Henderson, G., Cox, F., Kittelmann, S., Miri, V.H., Zethof, M., Noel, S.J., Waghorn, G.C.,
654 Janssen, P.H., 2013. Effect of DNA extraction methods and sampling techniques on the
655 apparent structure of cow and sheep rumen microbial communities. PLoS One 8,
656 e74787. https://doi.org/10.1371/journal.pone.0074787

657 Hoover, W.H., Stokes, S.R., 1991. Balancing carbohydrates and proteins for optimum rumen
658 microbial yield. J. Dairy Sci. 74, 3630–3644. https://doi.org/10.3168/jds.S0022-
659 0302(91)78553-6
660 Hristov, A. N., Hanigan, M., Cole, A., Todd, R., McAllister T. A., Ndegwa, P. and Rotz, A.,
661 2011. Review: Ammonia emissions from dairy farms and beef feedlots. Can. J. Anim.
662 Sci. 91, 1–35. https://doi.org/10.4141/CJAS10034
663 Jami, E., Israel, A., Kotser, A., Mizrahi, I., 2013. Exploring the bovine rumen bacterial
664 community from birth to adulthood. ISME J. 7, 1069–1079.
665 https://doi.org/10.1038/ismej.2013.2
666 Jouany, J.P., 1982. Volatile fatty acid and alcohol determination in digestive contents, silage
667 juices, bacterial cultures and anaerobic fermentor contents. Sci. Aliments 2, 131-144.
668 Kittelmann, S., Seedorf, H., Walters, W.A., Clemente, J.C., Knight, R., Gordon, J.I., Janssen,
669 P.H., 2013. Simultaneous amplicon sequencing to explore co-occurrence patterns of
670 bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities.
671 PLoS One 8, e47879. https://doi.org/10.1371/journal.pone.0047879
672 Kraatz, M., Wallace, R.J., Svensson, L., 2011. *Olsenella umbonata* sp. nov., a
673 microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig
674 jejunum, and emended descriptions of *Olsenella*, *Olsenella uli* and *Olsenella profusa*.
675 Int. J. Syst. Evol. Microbiol. 61, 795–803. https://doi.org/10.1099/ijs.0.022954-0
676 Kumar, S., Indugu, N., Vecchiarelli, B., Pitta, D.W., 2015. Associative patterns among
677 anaerobic fungi, methanogenic archaea, and bacterial communities in response to
678 changes in diet and age in the rumen of dairy cows. Front. Microbiol.6, 781.
679 https://doi.org/10.3389/fmicb.2015.00781

La Reau, A.J., Meier-Kolthoff, J.P., Suen, G., 2016. Sequence-based analysis of the genus *Ruminococcus* resolves its phylogeny and reveals strong host association. Microb. Genomics 2. https://doi.org/10.1099/mgen.0.000099

Langenheder, S., Prosser, J.I., 2008. Resource availability influences the diversity of a functional group of heterotrophic soil bacteria. Environ. Microbiol. 10, 2245–2256. https://doi.org/10.1111/j.1462-2920.2008.01647.x

Liu, C., Meng, Q., Chen, Y., Xu, M., Shen, M., Gao, R., Gan, S., 2017a. Role of age-related shifts in rumen bacteria and methanogens in methane production in cattle. Front. Microbiol.8, 1563. https://doi.org/10.3389/fmicb.2017.01563

Liu, K., Xu, Q., Wang, L., Wang, J., Guo, W., Zhou, M., 2017b. The impact of diet on the composition and relative abundance of rumen microbes in goat. Asian-Australasian J. Anim. Sci. 30, 531–537. https://doi.org/10.5713/ajas.16.0353

Ludden, P.A., Cecava, M.J., 1995. Supplemental protein sources for steers fed corn-based diets: I. Ruminal characteristics and intestinal amino acid flows2. J. Anim. Sci. 73, 1466–1475. https://doi.org/10.2527/1995.7351466x

Luo, Z., Li, C., Cheng, Y., Hang, S., Zhu, W., 2015. Effects of low dietary protein on the metabolites and microbial communities in the caecal digesta of piglets. Arch. Anim. Nutr. 69, 212–226. https://doi.org/10.1080/1745039X.2015.1034521

Mackie, R.I., Gilchrist, F.M.C., Robberts, A.M., Hannah, P.E., Schwartz, H.M., 1978. Microbiological and chemical changes in the rumen during the stepwise adaptation of sheep to high concentrate diets. J. Agric. Sci. 90, 241. https://doi.org/10.1017/S0021859600055313

Magoč, T., Salzberg, S.L., 2011. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957–2963. https://doi.org/10.1093/bioinformatics/btr507

McCabe, M.S., Cormican, P., Keogh, K., O'Connor, A., O'Hara, E., Palladino, R.A., Kenny, D.A., Waters, S.M., 2015. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterized Succinivibrionaceae and an increase of the *Methanobrevibacter gottschalkii* clade in feed restricted cattle. PLoSOne 10, e0133234. https://doi.org/10.1371/journal.pone.0133234

Mills, L.S., Soulé, M.E., Doak, D.F., 1993. The keystone-species concept in ecology and conservation. Bioscience 43, 219–224. https://doi.org/10.2307/1312122

Morita, H., Shiratori, C., Murakami, M., Takami, H., Toh, H., Kato, Y., Nakajima, F., Takagi, M., Akita, H., Masaoka, T., Hattori, M., 2008. *Sharpea azabuensis* gen. nov., sp. nov., a Grampositive, strictly anaerobic bacterium isolated from the faeces of thoroughbred horses. Int. J. Syst. Evol. Microbiol. 58, 2682–2686. https://doi.org/10.1099/ijs.0.65543-0

Moya, A., Ferrer, M., 2016. Functional Redundancy-Induced Stability of Gut Microbiota Subjected to Disturbance. Trends Microbiol. 24, 402–413. https://doi.org/10.1016/J.TIM.2016.02.002

NRC., 2000. Nutrient requirements of beef cattle (Seventh Revised Edition: Update 2000). The National Academies Press, Washington, DC, USA.

Pacheco, A.R., Barile, D., Underwood, M.A., Mills, D.A., 2015. The impact of the milkglycobiome on the neonate gut microbiota. Annu. Rev. Anim. Biosci. 3, 419–445. https://doi.org/10.1146/annurev-animal-022114-111112

Peng, Y., Yu, K., Mu, C., Hang, S., Che, L., Zhu, W., 2017. Progressive response of large intestinal bacterial community and fermentation to the stepwise decrease of dietary crude protein level in growing pigs. Appl. Microbiol. Biotechnol. 101, 5415–5426. https://doi.org/10.1007/s00253-017-8285-6

729 Pereira, F.C., Berry, D., 2017. Microbial nutrient niches in the gut. Environ. Microbiol. 19,
730 1366–1378. https://doi.org/10.1111/1462-2920.13659
731 Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., McKinnon, J.J.,
732 McAllister, T.A., 2013.Characterization of the core rumen microbiome in cattle during
733 transition from forage to concentrate as well as during and after an acidotic challenge.
734 PLoSOne 8, e83424. https://doi.org/10.1371/journal.pone.0083424
735 Prins, R.A., Stewart, C.S., 1997. Microbial interactions in the rumen. Reprod. Nutr. Dev. 37,
736 1–4. https://doi.org/10.1051/rnd:19970701
737 R Core Team., 2018. R: A language and environment for statistical computing. R Foundation
738 for Statistical Computing, Vienna, Austria.
739 Reynal, S.M., Broderick, G.A., 2005. Effect of dietary level of rumen-degraded protein on
740 production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88, 4045–
741 4064. https://doi.org/10.3168/jds.S0022-0302(05)73090-3
742 Rosero, J.A., Killer, J., Sechovcová, H., Mrázek, J., Benada, O., Fliegerová, K., Havlík, J.,
743 Kopečný, J., 2016. Reclassification of *Eubacterium rectale* (Hauduroy et al. 1937)
744 prévot 1938 in a new genus *Agathobacter* gen. nov.as*Agathobacter rectalis* comb. nov.,
745 and description of *Agathobacter ruminis* sp. nov., isolated from the rumen contents of
746 sheep and cows. Int. J. Syst. Evol. Microbiol. 66, 768–773.
747 https://doi.org/10.1099/ijsem.0.000788
748 Rotger, A., Ferret, A., Calsamiglia, S., Manteca, X., 2006. Effects of nonstructural
749 carbohydrates and protein sources on intake, apparent total tract digestibility, and
750 ruminal metabolism in vivo and in vitro with high-concentrate beef cattle diets. J. Anim.
751 Sci. 84, 1188–1196. https://doi.org/10.2527/2006.8451188x

752 Russell, J.B., Sniffen, C.J., Van Soest, P.J., 1983. Effect of carbohydrate limitation on
753 degradation and utilization of casein by mixed rumen bacteria. J. Dairy Sci. 66, 763–
754 775. https://doi.org/10.3168/jds.S0022-0302(83)81856-6
755 Satter, L.D., Slyter, L.L., 1974. Effect of ammonia concentration on rumen microbial protein
756 production in vitro. Br. J. Nutr. 32, 199. https://doi.org/10.1079/BJN19740073
757 Segers, J.R., Faulkner, D.B., Retallick, K.M., Shike, D.W., 2014. Effects of protein and fat
758 concentration in coproduct-based growing calf diets on performance and carcass
759 composition. J. Anim. Sci. 92, 5603–5611. https://doi.org/10.2527/jas.2014-7880
760 Shabat, S.K.B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller,
761 M.E., White, B.A., Shterzer, N., Mizrahi, I., 2016. Specific microbiome-dependent
762 mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10, 2958–
763 2972. https://doi.org/10.1038/ismej.2016.62
764 Shrivastava, V.S., Talapatra, S.K., 1962. Pasture studies in Uttar Pradesh II. Use of some
765 natural indicators to determine the plane of nutrition of a grazing animal. Indian J. Dairy
766 Sci. 15, 154–160.
767 Song, M.K., Kennelly, J.J., 1990. Ruminal fermentation pattern, bacterial population and
768 ruminal degradation of feed ingredients as influenced by ruminal ammonia
769 concentration. J. Anim. Sci. 68, 1110–1120. https://doi.org/10.2527/1990.6841110x
770 Stevenson, D.M., Weimer, P.J., 2007. Dominance of *Prevotella* and low abundance of
771 classical ruminal bacterial species in the bovine rumen revealed by relative
772 quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165–174.
773 https://doi.org/10.1007/s00253-006-0802-y
774 Šuľák, M., Sikorová, L., Jankuvová, J., Javorský, P., Pristaš, P., 2012. Variability of
775 *Actinobacteria*, a minor component of rumen microflora. Folia Microbiol.(Praha). 57,
776 351–353. https://doi.org/10.1007/s12223-012-0140-7

777 Tapio, I., Fischer, D., Blasco, L., Tapio, M., Wallace, R.J., Bayat, A.R., Ventto, L., Kahala,
778 M., Negussie, E., Shingfield, K.J., Vilkki, J., 2017. Taxon abundance, diversity, co-
779 occurrence and network analysis of the ruminal microbiota in response to dietary
780 changes in dairy cows. PLoS One 12, e0180260.
781 https://doi.org/10.1371/journal.pone.0180260
782 Van Bruchen, J., Rouwers, S.M., Bandma, G.A., Leffering, C.P., 1985. Digestion of proteins
783 of varying degradability sheep. 1. Fermentation and rate of passage from the
784 reticulorumen. Neth. J. Agric. Sci. 33, 263-272.
785 Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fibre, neutral
786 detergent fiber and non-starch polysaccharides in relation to animal nutrition. J. Dairy
787 Sci. 74, 3583-3597.
788 Verdú, M., Bach, A., Devant, M., 2017. Effect of feeder design and concentrate presentation
789 form on performance, carcass characteristics, and behavior of fattening Holstein bulls
790 fed high-concentrate diets. Anim. Feed Sci. Technol. 232, 148–159.
791 https://doi.org/10.1016/J.ANIFEEDSCI.2017.07.003
792 Wang, C., Liu, Q., Guo, G., Huo, W.J., Liang, Y., Pei, C.X., Zhang, S.L., Yang, W.Z., Wang,
793 H., 2017. Effects of different dietary protein levels and rumen-protected folic acid on
794 ruminal fermentation, degradability, bacterial populations and urinary excretion of
795 purine derivatives in beef steers. J. Agric. Sci. 155, 1477–1486.
796 https://doi.org/10.1017/S0021859617000533
797 Weimer, P.J., 2015. Redundancy, resilience, and host specificity of the ruminal microbiota:
798 implications for engineering improved ruminal fermentations. Front. Microbiol. 6, 296.
799 https://doi.org/10.3389/fmicb.2015.00296

800 Williams, A.P., Cockburn, J.E., 1991. Effect of slowly and rapidly degraded protein sources
801 on the concentrations of amino acids and peptides in the rumen of steers. J. Sci. Food
802 Agric. 56, 303–314. https://doi.org/10.1002/jsfa.2740560306
803 Yang, C., Si, B., Diao, Q., Jin, H., Zeng, S., Tu, Y., 2016. Rumen fermentation and bacterial
804 communities in weaned Chahaer lambs on diets with different protein levels. J. Integr.
805 Agric. 15, 1564–1574. https://doi.org/10.1016/S2095-3119(15)61217-5
806 Zhang, B., Wang, C., Liu, H., Liu, J., Liu, H., 2017. Effects of dietary protein level on growth
807 performance and nitrogen excretion of dairy heifers. Asian-Australasian J. Anim. Sci.
808 30, 386–391. https://doi.org/10.5713/ajas.16.0214

809 ***Table 1*** *Ingredients and chemical average composition of concentrates and straw.*

Item[1]	Control concentrate[2]	Low protein concentrate[2]	Straw
Ingredient composition, g/kg FM			
Raw corn	431.2	527.1	
Bran	204.5	248.8	
Raw barley	99.5	21.0	
Corn DDGS	65.0	0.0	
Wet ear of corn silage	69.8	49.8	
Palm kernel meal	30.0	73.5	
Palm kernel oil	23.6	21.1	
Calcium carbonate	26.9	18.5	
Sugarcane molasses	18.4	17.2	
Soybean hunks	15.6	10.0	
Sodium bicarbonate	7.8	7.5	
White salt	3.5	3.8	
Urea	2.5	0.0	
Vitamin/mineral premix	2.0	2.0	
Chemical composition, g/kg DM[3]			
DM, g/kg FM	856.2	862.1	857.2
OM	939.9	943.3	842.9
CP	140.7	117.7	71.9
EE	51.4	72.0	14.2
NDF	202.5	207.1	442.1
ADF	70.7	78.5	281.8
Nutrient composition			
UFC , UFC/kg DM	1.00	0.97	0.36
PDIN, g/kg DM	89.75	79.80	40.83
PDIE, g/kg DM	89.90	86.20	52.61
PDIN/UFC	90.29	82.64	113.42
PDIE/UFC	90.44	88.58	146.14

810 [1] ADF: acid detergent fiber; CP: crude protein; DDGS: distillers dried grains and solubles;
811 DM: dry matter; EE: ether extract; FM: fresh matter; NDF: neutral detergent fiber; OM:
812 organic matter; PDIN and PDIE: protein digestible in the small intestine allowed by protein
813 and energy, respectively; UFC: forage unit for meat production.

814 [2] Concentrate was presented in form of expanded pellets.

815 [3] See "Chemical analyses" in Materials and Methods section for further information about
816 chemical composition analyses.

817 ***Table 2*** *Dry matter intake, nutrient apparent digestibility and nitrogen balance.*
818

Item [1,2]	GRO		FIN		SEM	P-value		
	CTR	LP	CTR	LP		Treatment	Period	TxP
n	10	10	10	8				
Intake								
DM, kg/d	6.46^{b}	5.65^{b}	9.45^{a}	9.69^{a}	0.345	0.460	<0.001	0.049
Concentrate DM	5.71^{b}	4.87^{b}	8.08^{a}	8.25^{a}	0.266	0.328	<0.001	0.002
Straw DM	0.76	0.78	1.36	1.37	0.228	0.937	0.009	0.973
Apparent digestibility coefficient								
DM	0.737	0.745	0.744	0.722	0.014	0.654	0.448	0.183
OM	0.747	0.750	0.753	0.729	0.015	0.526	0.549	0.254
CP	0.717^{ab}	0.711^{ab}	0.725^{a}	0.673^{b}	0.012	0.042	0.099	0.014
N balance								
N intake, g/d	135.00^{c}	97.63^{d}	200.97^{a}	176.79^{b}	6.173	<0.001	<0.001	0.105
N excretion, g/d	66.68^{b}	47.06^{c}	94.54^{a}	85.47^{a}	5.001	0.017	<0.001	0.172
Feces	38.02^{b}	28.17^{c}	55.35^{a}	57.50^{a}	2.542	0.175	<0.001	0.006
Urine	28.65^{ab}	18.88^{b}	39.19^{a}	30.42^{ab}	3.663	0.010	0.002	0.882

819 [1] Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280
820 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low
821 protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Standard error of the mean (SEM) and significance of treatment,
822 period and their interaction (TxP) effects are shown.

823 [2] CP: crude protein; DM: dry matter; N: nitrogen; OM: organic matter.

824 [a-d] Mean values within a row with unlike superscript letters differ ($P < 0.05$).

825 ***Table 3*** *Ruminal fermentation parameters.*

Item[1,2]	GRO		FIN		SEM	P-value		
	CTR	LP	CTR	LP		Treatment	Period	TxP
n	10	10	9	9				
Ruminal fermentation parameters								
pH	7.29[a]	7.28[a]	6.45[b]	6.90[ab]	0.124	0.080	<0.001	0.065
Ammonia-N, mg/L	4.15	0.58	6.30	7.06	1.985	0.511	0.026	0.235
VFA, mmol/L	66.96[ab]	57.81[b]	91.27[a]	60.98[b]	7.796	0.033	0.050	0.123
VFA, mol/100 mol								
Acetate	46.30	47.79	45.51	45.94	0.769	0.289	0.044	0.392
Propionate	43.59[a]	39.46[b]	42.87[a]	42.73[a]	0.783	0.022	0.077	0.009
Butyrate	6.42[c]	9.27[a]	7.57[b]	7.03[bc]	0.228	<0.001	0.019	<0.001
Ratio A/P	1.07[b]	1.22[a]	1.07[b]	1.08[b]	0.039	0.086	0.042	0.051

826 [1] Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280
827 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low
828 protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Standard error of the mean (SEM) and significance of treatment,
829 period and their interaction (TxP) effects are shown.

830 [2] Ammonia-N: ammonia nitrogen; A/P: acetate/propionate; VFA: volatile fatty acids.

831 [a-c] Mean values within a row with unlike superscript letters differ ($P < 0.05$).

832 ***Table 4*** *Main phyla and genera abundance in ruminal fluid microbiota and microbial alpha diversity.*

Item[1]	GRO		FIN		SEM	Adjusted P-value		
	CTR	LP	CTR	LP		Treatment	Period	TxP
n	10	9	10	9				
Phyla abundance,%								
Bacteroidetes	85.60[a]	82.05[a]	66.20[b]	79.11[ab]	2.529	0.302	0.002	0.016
Firmicutes	11.48[b]	10.99[b]	26.20[a]	14.30[b]	1.845	0.051	0.001	0.016
Actinobacteria	2.37	6.42	5.78	5.42	0.881	0.166	0.438	0.083
Euryarchaeota	0.19	0.15	0.85	0.39	0.212	0.459	0.166	0.512
Proteobacteria	0.19	0.30	0.33	0.68	0.113	0.205	0.098	0.459
Fibrobacteres	0.12	0.04	0.48	0.02	0.215	0.459	0.544	0.512
Genera abundance,%								
Prevotella	85.56[a]	81.74[a]	66.16[b]	79.02[ab]	2.549	0.428	0.006	0.036
Roseburia	2.77[b]	2.36[b]	8.27[a]	5.39[ab]	0.712	0.236	0.002	0.386
Olsenella	0.90[b]	0.80[b]	3.16[a]	2.93[a]	0.235	0.617	<0.001	0.854
Selenomonas	2.53[a]	1.59[ab]	1.43[b]	1.48[ab]	0.197	0.319	0.037	0.105
Bifidobacterium	0.98[b]	5.18[a]	0.25[b]	0.52[b]	0.689	0.085	0.019	0.104
Agathobacter	0.88[b]	1.15[b]	2.59[a]	1.57[ab]	0.211	0.472	0.001	0.023
Sharpea	0.95[b]	0.06[b]	3.99[a]	0.21[b]	0.543	0.006	0.082	0.129
Ruminococcus	0.63[b]	0.29[b]	2.60[a]	1.23[ab]	0.379	0.346	0.006	0.355
Pseudobutyrivibrio	0.37	1.21	1.89	0.78	0.665	0.917	0.492	0.388
Microbial alpha diversity								
Richness	96.40	113.89	110.40	117.44	9.380	0.237	0.299	0.532
Shannon index	1.03[b]	1.73[a]	1.92[a]	1.66[a]	0.135	0.157	0.002	0.001
Simpson index	0.34[b]	0.65[a]	0.63[a]	0.55[a]	0.046	0.030	0.035	<0.001

833 [1] Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280

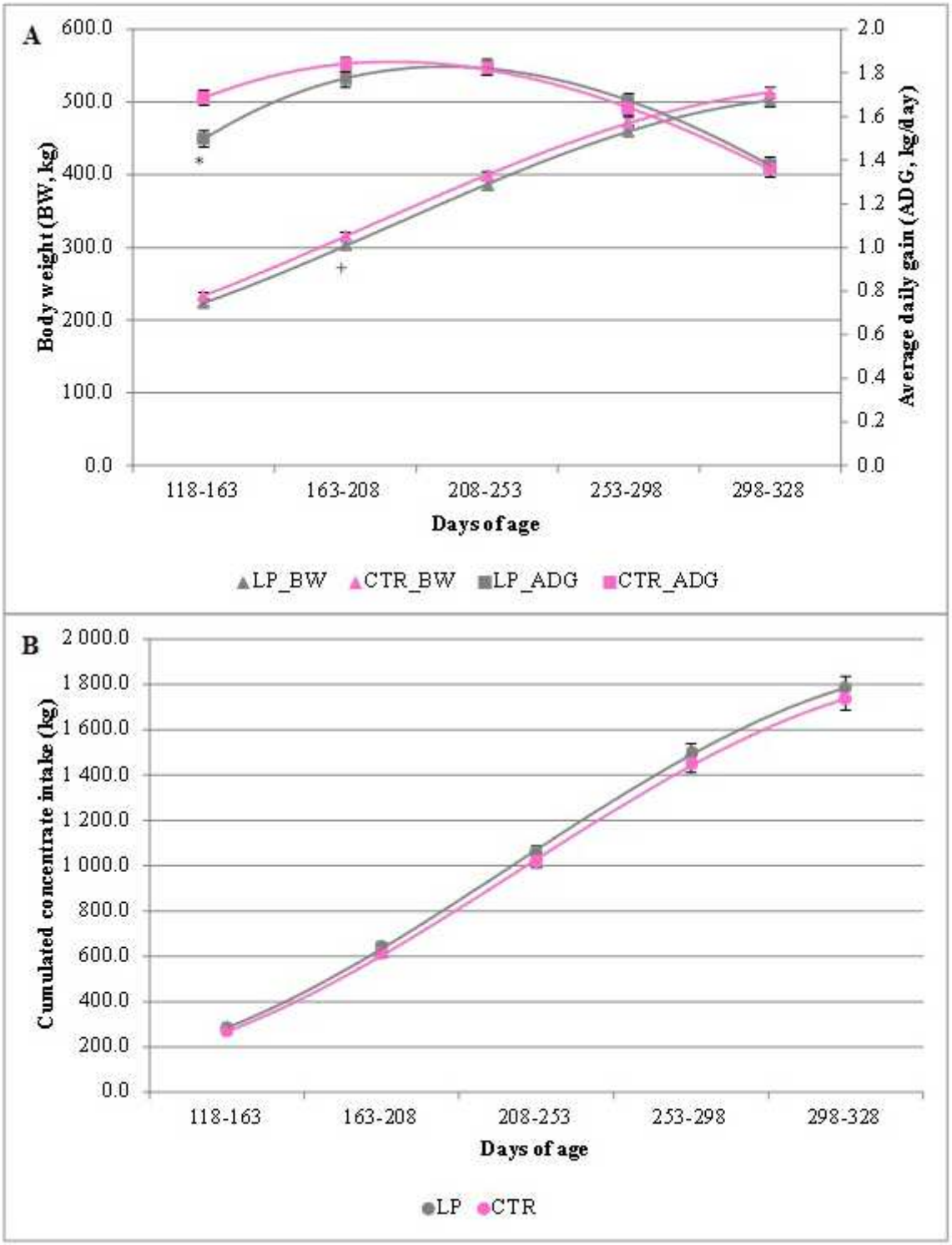
834 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low

835 protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Standard error of the mean (SEM) and significance of treatment,

836 period and their interaction (TxP) effects are shown.

837 [a-b] Mean values within a row with unlike superscript letters differ ($P<0.05$).

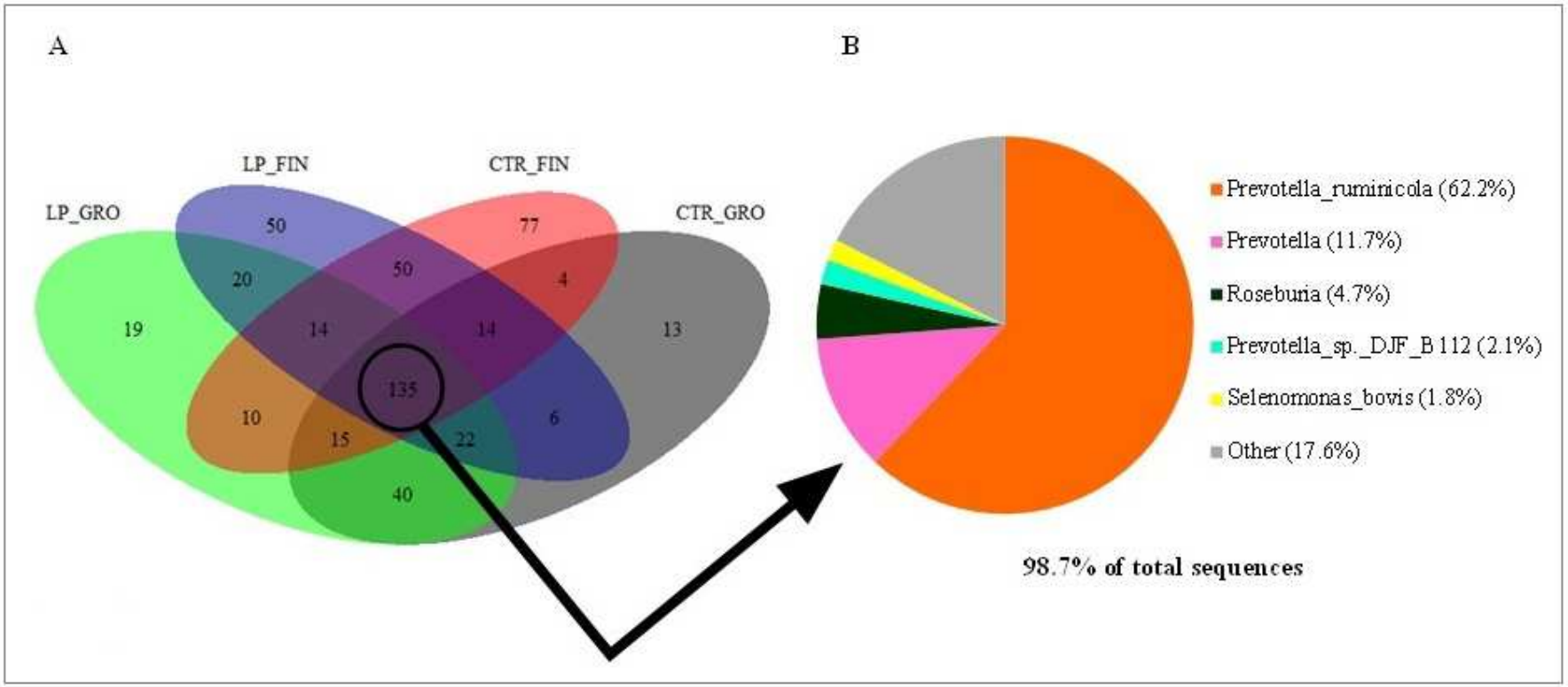
838 ***Figure 1**.Performance and concentrate intake evolution*[1].



839 [1] **(A)** Body weight and average daily gain and **(B)** cumulated concentrate intake obtained in
840 intensively reared Holstein bulls (from 118 to 328 d of age). Animals were fed two
841 concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP:

842 120 g/kg crude protein on a dry matter basis) plus barley straw.* means that a statistical
843 difference were found (P<0.05) and + means that a statistical tendency was found (P<0.10).

844 ***Figure 2*** *Distribution of bacterial and archaeal OTUs in ruminal fluid*[1].

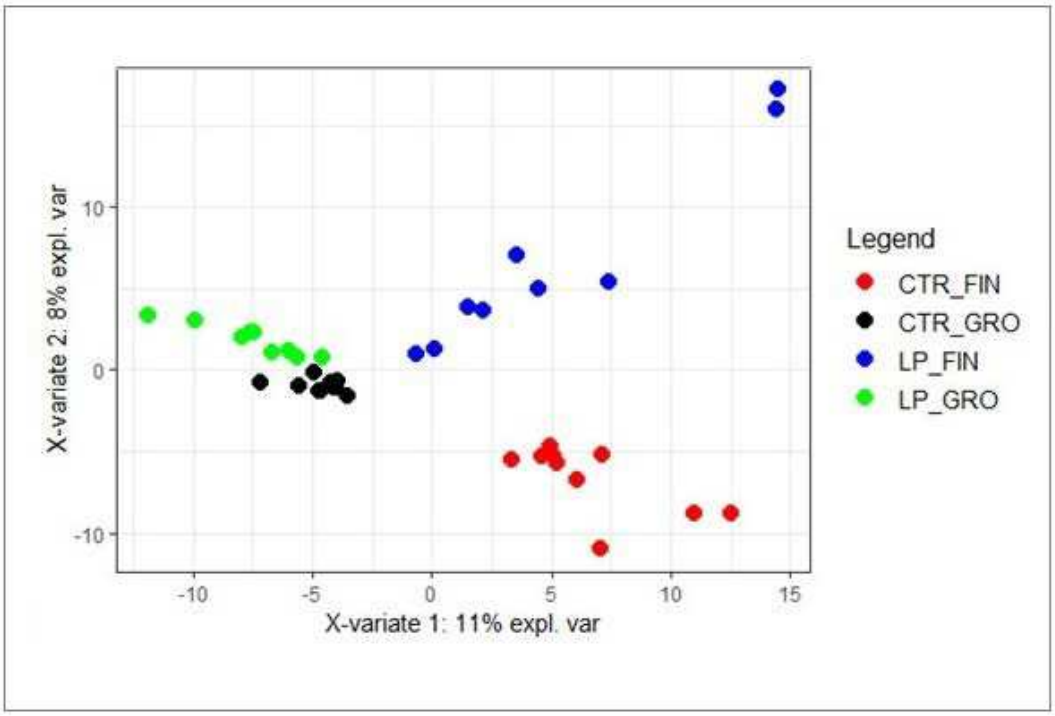


845 [1] Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280
846 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low
847 protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. **(A)** The Venn diagram shows the numbers of OTUs that were shared

848 or unshared by treatments and periods, depending of overlaps. **(B)** The pie diagram shows the core bacterial and archaeal composition within the

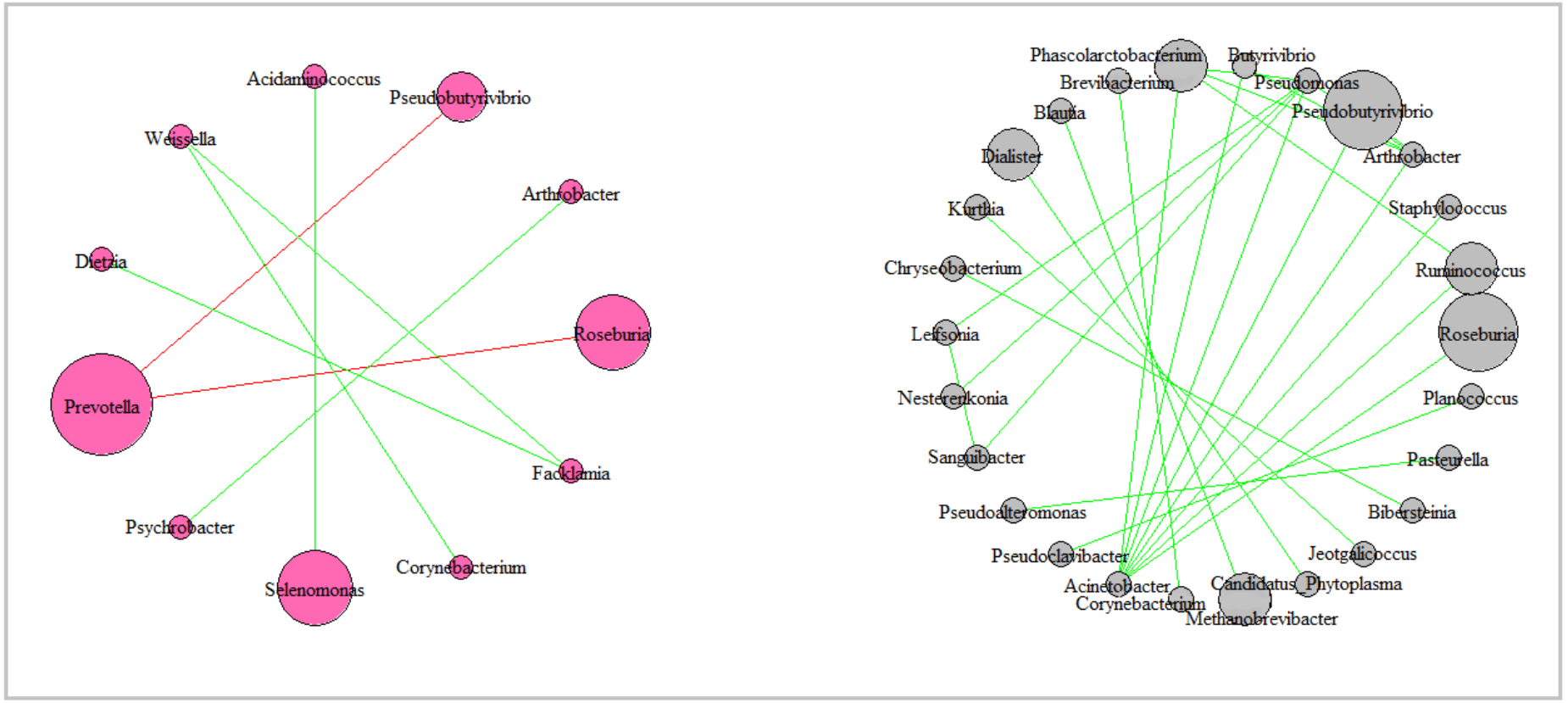
849 four groups (OTUs that occurred at low abundance were included as “other”).

850 ***Figure 3*** *Partial Least Squares-Discriminant Analysis of ruminal fluid microbiota*[1].



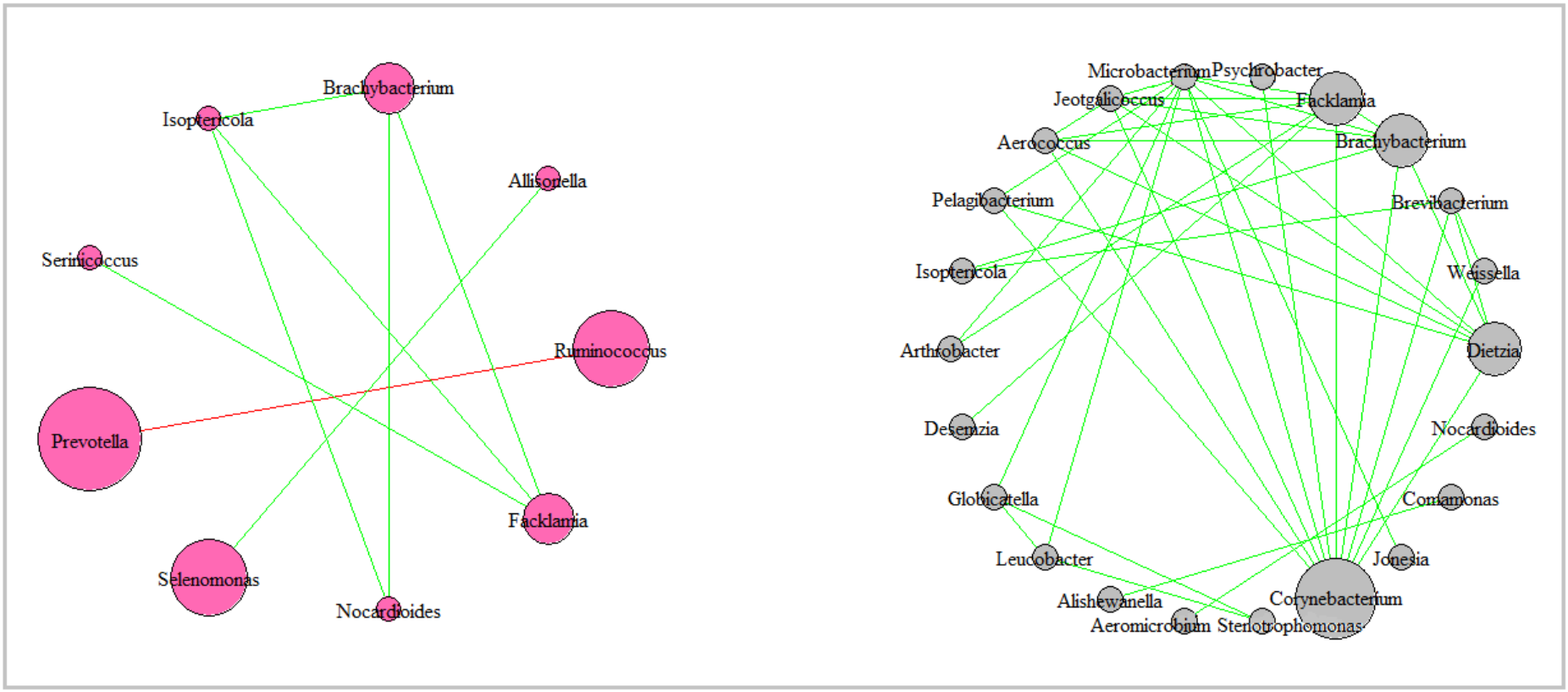
851 [1] Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age
852 and 225 kg of body weight) and finishing (FIN: 280 d of age and 444 kg of body weight).
853 Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter
854 basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Each
855 point represents a different sample and a greater distance between two points infers a higher
856 dissimilarity between them.

Figure 4 *Microbial networks in the rumen of growing cattle*[1].



[1] Obtained in intensively reared Holstein bulls (160 d of age and 225 kg of body weight). Animals were fed two concentrates: control (**A**, 140 g/kg crude protein on a dry matter basis) or low protein (**B**, 120 g/kg crude protein on a dry matter basis) plus barley straw. Networks were generated based on those genera which significantly correlated ($r>0.60$ and $P<0.05$); green and red arrows indicate positive and negative correlations, respectively.

862 ***Figure 5*** *Microbial networks in the rumen of finishing cattle*[1].



863 [1] Obtained in intensively reared Holstein bulls (280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (**A**, 140
864 g/kg crude protein on a dry matter basis) or low protein (**B**, 120 g/kg crude protein on a dry matter basis) plus barley straw. Networks were
865 generated based on those genera which significantly correlated ($r>0.60$ and $P<0.05$); green and red arrows indicate positive and negative
866 correlations, respectively.

867 **SUPPLEMENTAL MATERIAL**

868 ***Supplementary Table 1*** *Correlation between ruminal bacterial and archaeal taxa in growing cattle.*

Phylum	Genera	CORRELATIONS[1,2]											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Actinobacteria	*Arthrobacter*						-0.727		0.816		-0.816		-0.692
	Bifidobacterium[3]											0.709	
	Corynebacterium	0.672											
	Dietzia	0.793											
	Leifsonia					0.711			0.837		-0.837		
	Nesterenkonia										-0.940		
	Prauserella								0.693				
	Sanguibacter								0.822		-0.822		
Bacteroidetes	*Bacteroides*[3]	-0.638		-0.723			-0.767				-0.833		
	Chryseobacterium								0.693				
	Prevotella							-0.782		0.818	0.700	-0.721	
Fibrobacteres	*Fibrobacter*							0.854		-0.896			
Firmicutes	*Agathobacter*						-0.733		0.717		-0.750		
	Butyrivibrio						-0.792		0.911		-0.690		-0.885
	Clostridium		0.686				-0.929		0.828	-0.719			-0.828
	Desemzia							0.664		-0.710			
	Dialister							0.663		-0.644			0.700
	Eubacterium							0.661		-0.685		0.770	
	Facklamia	0.652											
	Phascolarctobacterium						-0.883		0.953		-0.831		-0.822
	Planomicrobium						-0.730		0.707				

869

870 ***Supplementary Table 1*** *Continued*

Phylum	Genera	CORRELATIONS[1]											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Firmicutes	*Roseburia*						-0.800	0.745	0.733	-0.770	-0.733	0.721	
	Ruminococcus						-0.917	0.939	0.933	-0.927			-0.933
	Sharpea [3]									-0.685			
	Staphylococcus						-0.681		0.792		-0.800		
	Streptococcus					0.731	-0.738						-0.738
	Weissella[3]	0.759									-0.732		
Proteobacteria	*Acinetobacter*						-0.863		0.932		-0.794		-0.794
	Alysiella		0.667										
	Brevundimonas						-0.671		0.820				-0.783
	Pseudomonas								0.817		-0.908	-0.674	
	Psychrobacter								0.678				
	Stenotrophomonas												-0.725

871

872 [1] Obtained in intensively reared Holstein bulls (160 days of age and 225 kg of body weight). Animals were fed two concentrates: control (CTR:

873 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw.

874 [2] Only Spearman correlations with r>0.60 and P<0.05 are shown (n=19). Green and red values indicate positive and negative correlations,

875 respectively.

876 [3] Genus with statistically different ruminal abundance (P<0.05) between CTR and LP fed animals.

877 ***Supplementary Table 2*** *Correlation between ruminal bacterial and archaeal taxa in finishing cattle.*

Phylum	Genera	CORRELATIONS[1,2]											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Actinobacteria	*Bifidobacterium*[3]							-0.748		0.632			
	Georgenia	0.757											
	Kocuria									0.658			
	Microbacterium									-0.722			
	Rothia				0.820								
	Serinicoccus									-0.662			
Bacteroidetes	*Bacteroides*[3]			0.707		-0.683							
	Prevotella									0.794			
Euryarchaeota	*Methanobrevibacter*			0.675			0.736					0.782	
Firmicutes	*Agathobacter*								0.867		-0.750		
	Bacillus							-0.833		0.768		-0.638	
	Dialister												
	Eubacterium		0.723								-0.733		
	Globicatellla			0.733									
	Jeotgalicoccus									-0.665			
	Mitsuokella[3]								-0.683				
	Planococcus		-0.729										
	Planomicrobium	0.737											
	Pseudobutyrivibrio									-0.693	-0.733		0.767
	Roseburia	-0.644									-0.733		
	Ruminococcus									-0.758			
	Selenomonas							-0.636					
Proteobacteria	*Alysiella*		0.698		0.935		-0.811						

878

879 ***Supplementary Table 2*** *Continued*

Phylum	Genera	CORRELATIONS[1]											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Proteobacteria	*Bibersteinia*										-0.694		
	Halomonas											0.642	
	Paracoccus	0.739		0.694		-0.850							
	Pseudaminobacter											0.655	
	Pseudomonas								-0.766				
Spirochaetes	*Treponema*							0.679		-0.673			

880

881 [1] Obtained in intensively reared Holstein bulls (280 days of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR:

882 140 g/kg crude protein on a dry matter basis) or low protein (LP: 12 g/kg crude protein on a dry matter basis) plus barley straw.

883 [2] Only Spearman correlations with r>0.60 and P<0.05 are shown (n=19). Green and red values indicate positive and negative correlations,

884 respectively.

885 [3] Genus with statistically different ruminal abundance (P<0.05) between CTR and LP fed animals.

886

887