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# **Metagenomics Analyses of Cellulose and Volatile Fatty Acids Metabolism by Microorganisms in the Cow Rumen**

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## 29 **ABSTRACT**

30 The purpose of this study was to evaluate the effects of different forage-to-concentrate  
31 (F:C) ratios (7:3 high-forage, 3:7 high-concentrate) on rumen microflora and fiber  
32 degradation mechanism. Compared with the high-concentrate (HC) group, the  
33 high-forage(HF) group showed improved fiber degradation and a sustained high level  
34 of carboxymethyl cellulose (CMCase),  $\beta$ -glucosidase and  $\beta$ -xylosidase activities, but  
35 the total VFAs decreased. Among bacteria at the family level, *Lachnospiraceae* and  
36 *Succinivibrionaceae* in HF groups were 2-fold and 4-fold more abundant than in the  
37 HC group, respectively. A KEGG analysis revealed that succinate-CoA synthetase  
38 (EC: 6.2.1.5) and propionate-CoA transferase (EC: 2.8.3.1) leading directly to  
39 propionate production were more abundant in HC group. Conversely, butyryl-CoA  
40 dehydrogenase (EC: 1.3.8.1) was directly related to butyrate production and was  
41 higher in the HF group. A gene expression analysis showed that the relative content of  
42 *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* was higher in the HF group  
43 and contributed more to fiber degradation and VFA production. *Prevotella ruminicola*,  
44 *Selenomonas ruminantium*, and *Veillonella alcalescens* contributed more to starch  
45 degradation and propionate production, which relative content was higher in the HC  
46 group. This research gave a further explanation of the fiber degradation parameters  
47 and microbiota under different F:C ration. The fiber-degrading bacteria in the  
48 roughage group have a high content level, and the corresponding cellulase activity is  
49 also high. These results supported the potential of diets for microbial manipulation,  
50 which can increase feed digestibility and explored new fibrinolytic bacteria.

51

## 52 **IMPORTANCE**

53 The forage of the cow's feed occupies a large proportion. The shortage of high-quality  
54 forage in cow breeding has become an important factor limiting the China's dairy  
55 industry. The effective measure is to improve the utilization of low-quality forage.  
56 Based on traditional nutrient metabolism, the reasons for the effects of roughage on  
57 the growth and metabolism of dairy cows can be explored, but the metabolic  
58 mechanism is not well analyzed, and the further utilization of forage is also limited.

59 Metagenomics has proven to be a powerful tool for studying rumen microbial  
60 structures and gene function. This experiment used metagenomics to study the  
61 metabolism of cellulose and volatile acids in the rumen. Our research showed that  
62 different forage-to-concentrate shifted the composition of microorganisms and the  
63 activity of enzymes, resulting in different metabolic pathways of volatile fatty acids.  
64 This work provides a background for microbial community composition and further  
65 use of forage.

66

67 **Key words:** forage-to-concentrate ratio, fiber decomposition, volatile fatty acid  
68 production, fibrolytic bacteria, gene expression

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## 71 1、 INTRODUCTION

72 The rumen ecosystem is recognized as a natural bioreactor for highly efficient  
73 degradation of fibers, and rumen microbes have an important effect on fiber  
74 degradation (1). The rumen provides anaerobic conditions and redox potentials that  
75 favor microbial growth and expression of fiber-degrading enzymes, which allows  
76 rumen microorganisms to breakdown cellulosic plant materials to meet ruminants'  
77 daily energy requirements by producing volatile fatty acids (VFAs). The degradation  
78 of carbohydrates in the rumen can be divided into two stages Fig.1 (1, 2). For example,  
79 cellulose is first hydrolyzed into cellobiose by ruminal microorganisms and then is  
80 further hydrolyzed to glucose, after which the glucose continues to be fermented to  
81 produce pyruvate (3-5). Finally, a series of fermentation reactions produce VFAs (6).  
82 These processes are all performed through the actions of rumen microbial enzymes.

83 The changes of F:C ratios in the diet significantly affect the number and type of  
84 rumen microorganisms and then affect the end products of fermentation (7, 8).  
85 Previous studies show that the content of *Fibrobacter* and *Ruminococcus* (both are  
86 mainly Fibrinolytic bacteria) increased when dietary fiber increased in the rumen (9,  
87 10). The type of diet directly causes changes in the rumen environment, causing  
88 changes in the rumen microbial population, population structure and enzyme activity.

89 Mcallan et al. (1994) and Ribeiro et al.(2015) (11, 12) studied the effects of rumen  
90 fermentation on cows with high-forage and high-concentrate diets, with the results  
91 indicating that total VFA and NH<sub>3</sub>-N were not affected. When concentrates in the diet  
92 increased, the acetate and butyrate were significantly reduced, but the propionate  
93 significantly increased.

94 Although much work has been done to investigate the effects of F:C in the diet of  
95 dairy animals on metabolism, most of the studies focused on the metabolism of  
96 nutrients or the production of volatile acids, which does not provide a systematic  
97 change, i.e., from cellulose fermentation to end-product VFAs and the variation of the  
98 microbial population during fermentation. The ruminal microbial ecosystem is an  
99 effective model for degrading fiber; therefore, it is important to understand how  
100 ecosystems develop and operate when shifting the diet of the host. Therefore, we  
101 combine metabolism and metagenomics to explore the changes from cellulose to end  
102 product VFAs and the changes of cellulose in rumen. At the same time, we also  
103 quantified major functional bacterial species using quantitative PCR to assess the role  
104 of these organisms in adapting to different diets (HC and HF).

105 However, we studied how rumen bacteria change and adapt to different rumen  
106 environments in different diets. Therefore, it is of practical significance to study the  
107 competition between fibrinolytic bacteria (the result of competition and its internal  
108 mechanism).

## 109 **2、 MATERIALS and METHODS**

### 110 **2.1 Experimental Design, Animals and Sample Collection**

111 Twelve ruminally cannulated, lactating Holstein cows averaging  $3.2 \pm 0.70$  (mean  $\pm$   
112 SE) years of age (range = 0.9 years) were used in this experiment. Cows were placed  
113 in individual tie stalls in a temperature-controlled room. Six animals (lactating cow)  
114 were fed a F:C ratio of 7:3, and the other six animals (dry cow) were fed a F:C ratio of  
115 3:7. Diet composition and nutrients were shown in Supplement Tab. 1. Animals were  
116 fed once daily at 8:00 h and allowed ad libitum consumption at 110% of the expected  
117 intake for four weeks before being sampled.

### 118 **Compliance with Ethical Standards**

119 **Conflicts of interest** All authors declare that they have no conflict of interest.

120 **Ethics approval** All animal studies were conducted according to the animal care and  
121 use guidelines of the Animal Care and Use Committee of Animal Science and  
122 Technology College, Northeast Agricultural University.

## 123 **2.2 Sample Collection and Measurements**

124 Rumen content samples were collected 4 h after feeding via a ruminal fistula.  
125 Representative rumen content samples were collected from each animal, and the solid  
126 from the liquid through four layers of cheesecloth were sampled. One part of each  
127 homogenized pellet was mixed with RNeasy lysis buffer (Qiagen, Crawley, UK), a reagent that  
128 protects and stabilizes cellular RNA. One part of each homogenized pellet with  
129 freshly prepared metaphosphoric acid (25% w/v; 1 mL) was added to 5 mL of filtered  
130 rumen fluid and vortexed, which was used to measure volatile fatty acids (VFAs).  
131 All samples were placed in liquid nitrogen within five min and were then taken to the  
132 laboratory and stored at -80°C until further testing.

133 For the determination of VFAs, samples with metaphosphoric acid were thawed at  
134 room temperature and then centrifuged (12,000 g for 15 min at 4°C). The supernatant  
135 was used to measure the VFAs. The VFA concentrations were determined by a  
136 capillary column gas chromatograph (13, 14).

137 For the enzyme activity assay, frozen pellets were thawed at room temperature. After  
138 being centrifuged at 3000g for 10 min (4°C), 10~15 ml of supernatant was taken for  
139 sonication (power 400W, crushed 3 times, 30S each time, 30S interval), and the  
140 crushed liquid was the sample to be tested. The assayed CMCase,  $\beta$ -glucosidase, and  
141 xylanase activity measured used the 3,5-dinitrosalicylic acid method (15, 16).

142 The glucose, cellobiose and xylose content in the samples were determined with  
143 high-performance liquid chromatography (HPLC, Waters 600, USA) using the  
144 Aminex HPX-87H column (Bio-Rad, America) and a refractive index detector  
145 (Waters 2414, USA) with 0.005 M H<sub>2</sub>SO<sub>4</sub> as the mobile phase, a column temperature  
146 of 60°C, and a velocity of 1.0 mL min<sup>-1</sup>, as assessed by a refractive index detector.

147 The cellulose, hemicellulose, lignin, neutral detergent fiber (NDF) and acid detergent  
148 fiber (ADF) content were analyzed using the Ankom A200 fiber analyzer (Ankom

149 Technology, Macedon, NY) using the method of Van Soest et al.(1991) (17). Briefly,  
150 the hemicellulose content was estimated as the difference between the NDF and the  
151 ADF, the cellulose content was estimated as the difference between the ADF and the  
152 acid-detergent lignin (ADL), and the lignin content was estimated as the difference  
153 between the ADL and the ash content.

## 154 **2.3 DNA and RNA extraction**

### 155 **2.3.1 DNA extraction**

156 Genomic DNA was extracted according to An et al.(2005)(18)and Minas et al.(2011)  
157 (19)with some improvements. DNA extraction was performed based on a  
158 CTAB-based DNA extraction method. The CTAB lysis buffer contained 2% w/v  
159 CTAB (Sigma-Aldrich, Poole, UK), 100 mM Tris–HCl (pH = 8.0; Fisher), 20 mM  
160 EDTA (pH = 8.0; Fisher) and 1.4 M NaCl (Fisher). The pH of the lysis buffer was  
161 adjusted to 5.0 prior to sterilization by autoclaving (20). The final DNA was  
162 resuspended in 100 µL TE buffer (pH = 8.0; Sigma-Aldrich) and stored at -80°C.

### 163 **2.3.2 RNA extraction, RNA Reverse Transcribed and qPCR primer design and** 164 **analysis**

165 RNA extraction was performed used the liquid nitrogen grinding + TRIzol reagent  
166 (Ambion, Carlsbad, USA); the steps are as described by Kang et al.(2009) and Wang  
167 et al.(2011) (21, 22) with some improvements. The RNA was reverse transcribed  
168 cDNA using a PrimeScript™ 1st strand cDNA Synthesis Kit (Code No. 6110A,  
169 TAKARA, Dalian, China), following the kit instructions. The reverse transcribed  
170 PCRs were as follows: 37°C for 15 min, 85°C for 5 sec, and 4°C for 10 min. cDNA  
171 stored the rest at -80°C. The PCR primers were listed in Tab.1 and were assembled  
172 from the literature (23, 24). Primers were provided by Sangon Biotech (Shanghai)  
173 Co.,Ltd (Shanghai, China).

174 The number of rumen microorganisms is expressed as a percentage relative to the  
175 total rumen 16Sr DNA: target bacteria (% total bacterial 16Sr DNA) =  $2^{-(Ct \text{ target bacteria} -$   
176  $Ct \text{ total bacteria})} \times 100\%$ , where target is the specific microbial group of interest.

## 177 **2.4 Deep Sequencing and KEGG Analysis**

178 Illumina TruSeq libraries were prepared from genomic DNA and sequenced on an

179 Illumina HiSeq 2500 instrument by Edinburgh Genomics. Five hundred bp paired-end  
180 reads were generated, resulting in between 8.08 and 10.09 Gb per sample (between  
181 65.84 and 83.68 million paired reads). For a functional analysis, classification  
182 functions were classified using the KEGG orthology database (version 67.1) to  
183 identify relationships between various pathways and obtain KEGG numbers and EC  
184 numbers. First, we matched the reads directly to KEGG genes, and the mismatch is  
185 allowed to be within 10%. All KEGG Orthologue groups (KO) with a hit equal to the  
186 best hit were examined. If we were unable to resolve the read to a single KO, the read  
187 is ignored; otherwise, the read was assigned to a unique KO. A statistical analysis was  
188 performed on each microorganism or function using the PROC GLM of SAS 9.4.

## 189 **2.6 Statistical Data Analyses**

190 Cellulose, hemicellulose and lignin were analyzed used covariance, and the content of  
191 each component in the diets was recorded as a covariate. The ANOVA statistical  
192 analyses of VFAs, pH, lactate, enzyme activity, sugar content and microbial diversity  
193 were done using the PROC GLM of SAS 9.4. The treatment was considered a fixed  
194 effect. Statistical significance was declared at  $P < 0.05$ .

## 195 **3、 RESULTS**

### 196 **3.1 Rumen fermentation parameters affected by different F:C ratio in diets.**

197 An overview of the analyses of in vivo fermentation, rumen pH, NH<sub>3</sub>-N, the content  
198 of VFA and lactate is provided in Tab.2. Rumen pH, NH<sub>3</sub>-N, the content of  
199 isobutyrate and lactate, and the ratio of acetate:propionate(A:P) showed a significant  
200 increase with the dietary forage level increase, while the content of TVFA, propionate,  
201 butyrate, valerate, and isovalerate significantly decreased ( $P < 0.01$ ). Acetate was the  
202 only fermentation product that did not differ significantly between the HF group and  
203 HC group ( $P = 0.59$ ). The content of TVFA in the rumen of the HC group was higher  
204 than that in the HF group, and the accumulation of VFA in the rumen resulted in a  
205 decrease in rumen pH. The concentration of NH<sub>3</sub>-N decreased significantly with the  
206 increase of concentrate in the diet.

### 207 **3.2 The composition of diets and rumen contents**

208 A covariance analysis was used to analyze the composition of rumen content in the  
209 HF and HC groups. The chemical composition of rumen contents was shown in Tab.3.  
210 The cellulose, hemicellulose, and acid detergent lignin (ADL) in the diets of HF group  
211 were significantly higher than in the HC group, but in the rumen content, there was no  
212 difference between two groups. In the rumen content, the HC group has a higher ( $P <$   
213 0.05) content of cellobiose (Fig.2A) and glucose (Fig.2B) compared to the HF group.  
214 The higher content of cellobiose and glucose indicate that the releasing rates of these  
215 sugars were faster from the HC group than from the HF group. This phenomenon is  
216 consistent with the  $\beta$ -glucosidase (Fig.2D), and the  $\beta$ -glucosidase activity in the HC  
217 group was higher than the HF group. The fiber digestibility of HC group was shown  
218 to be higher than of HF group, because the content of cellulose in the HC group was  
219 lower, and the content of the cellobiose decomposed by cellulose and glucose  
220 decomposed by cellobiose were higher than the HF group. Moreover, the  
221 carboxymethyl cellulase (CMCase) (Fig.2C) and  $\beta$ -glucosidase activity in the HC  
222 group were higher than in the HF group. The content of xylan and xylose were higher  
223 in HF group but not significantly so (Fig.3A,3B). The  $\beta$ -xylosidase in the HC group  
224 was significantly higher than in the HF group (Fig.3C).

### 225 **3.3 Ruminal microbial community changes within HC and HF groups**

226 At a threshold of  $>0.1\%$  relative abundance, 57 bacterial taxa (97% sequence  
227 similarity) were retrieved from the 16S rDNA gene amplicon sequences, while 52 taxa  
228 were retrieved from the metagenome genes. At the family level, the five taxa  
229 accounted for approximately 65% of the 16S rRNA gene amplicon sequences and the  
230 metagenome data in all samples: *Acidaminococcaceae*, *Lachnospiraceae*,  
231 *Prevotellaceae*, *Ruminococcaceae* and *Succinivibrionaceae* (Fig.4). Although there  
232 was no significant difference ( $P \leq 0.05$ ) in the abundance of *Ruminococcaceae*  
233 between HF and HC groups. All other taxa showing different abundance at the family  
234 level, *Lachnospiraceae* and *Succinivibrionaceae* being more abundant in the HF  
235 group, yet, *Prevotellaceae* and *Acidaminococcaceae* being more abundant in the HC  
236 group, that all based on both 16S rDNA amplicon and metagenomic sequencing data  
237 (Fig. 4a and 4b). At the genus-level resolution, eight taxa presented significantly



238 different relative abundances in the HF and HC groups based on GLM ( $P \leq 0.05$ ), four  
239 of which were more abundant in the HF group and four were more abundant in the  
240 HC group (Tab.4). The most noteworthy were *Prevotella* (family *Prevotellaceae*),  
241 *Selenomonas\_1* and *Veillonella* (both from the family *Acidaminococcaceae*), which  
242 were more abundant in the HF group based on both 16S rDNA and metagenome  
243 datasets and occupy 3.47, 2.42 and 1.43% of the metagenome reads from the HC  
244 group, respectively. *Ruminococcus* (family *Lachnospiraceae*) were the principal  
245 rumen cellulose-degrading bacteria and were significantly more abundant in the HF  
246 group in both datasets, with an average relative abundance of ~2% in these animals.  
247 *Prevotella* and *Selenomonas\_1* belong to the principal rumen starch-degrading  
248 bacteria. *Succinivibrio* and *Selenomonas* in the HC group were more enrichment may  
249 be related to the diet in HC group has more starch and maltose and less cellulose and  
250 xylan.

### 251 **3.4 Volatile fatty acid metabolism**

252 Volatile fatty acids act as energy sources for ruminants; therefore, it was important to  
253 study the metabolism of VFAs. The production of acetate was contained in the  
254 metabolic pathway of butyrate; therefore, the metabolism of propionate and butyrate  
255 was the main research focus.

#### 256 **3.4.1 Genes directly involved in propionate**

257 Many genes involved in the metabolism of short chain fatty acids are diverse and  
258 abundant in the metagenomic dataset, including genes encoding pyruvate  
259 fermentation to lactate and further fermentation to propionate, which transformation  
260 pyruvate to propionate, including the acrylate pathway and the succinate pathway.  
261 Plant polysaccharides are fermented by the rumen microbial, and finally, propionate is  
262 mainly production.

263 Genes encoding enzymes that are directly involved in propionate were analyzed for  
264 their abundance in HC and HF groups. Genes encoding the lactate-acrylate pathway  
265 produce propionate (Fig.S1). With the exception of the gene K01026 for lactyl  
266 transferase (EC:2.8.3.1), lower abundance in the HF group was found. The other  
267 genes, K00016 for acyl-CoA dehydratase (EC:1.3.8.7) and K00249 for lactate

268 dehydrogenase (EC:1.1.1.27), directly involved in propionate were higher in the HC  
269 group than in the HF group. However, in the lactate-acrylate pathway, the  
270 representative genes of lactoyl-CoA dehydratase (EC: 4.2.1.54) are not included in the  
271 KEGG gene dataset; therefore, this metabolic pathway cannot produce propionate.  
272 Conversely, fermenting to propionate seems to be more likely given the high readings  
273 of the genes involved via the succinate pathway. Such a pathway has been  
274 demonstrated in Fig.5. Genes involved in the succinate pathway to propionate showed  
275 significantly higher read counts in the HC group, which included genes K01902 and  
276 K01903 for succinate-CoA synthetase (EC:6.2.1.5) and gene K01026 for lactyl  
277 transferase (EC:2.8.3.1), both of which were predicted as being the limiting factors for  
278 propionate formation. Pyruvate carboxylase (EC 6.4.1.1) of genes K01958, K01959  
279 and K01960 was higher in the HF group than in the HC group. The other intermediate  
280 enzymes were all found, and the difference was not significant. VFA produced by  
281 rumen fermentation can be used as an energy supply for animals, of which only  
282 propionate gluconeogenesis is the main source of glucose. Therefore, propionate has  
283 important physiological significance for ruminants.

#### 284 **3.4.2 Genes directly involved in butyrate**

285 The genes encoding the butyrate formation pathway were analyzed for their  
286 abundance in the HC and HF groups (Fig.6). With the exception of gene K00248 for  
287 butyryl-CoA dehydrogenase (EC:1.3.8.1) and genes K01034 and K01035 for  
288 acetate-CoA transferase (EC:2.8.3.8), the content of all genes encoding the translation  
289 of pyruvate into butyrate in HC group showed higher or not significantly different  
290 than in HF group, at gene levels. The genes for acetoacetyl-CoA reductase  
291 (EC:1.1.1.36) and correlations to butyrate yield were not available in the KEGG  
292 database. The enzymes of butyryl-CoA dehydrogenase (EC:1.3.8.1) and acetate-CoA  
293 transferase (EC:2.8.3.8) were needed for the last two steps in the formation of  
294 butyrate, Therefore, the butyrate in the HF group should be higher than that in the HC  
295 group, which was consistent with the content of butyrate determination. No difference  
296 was found in Phosphate butyryltransferase (EC:2.3.1.19) between the HF and HC  
297 groups. Gene K00929 for butyrate kinase (EC:2.7.2.7) in the HC group was higher

298 than HF group. From fig.6, we can see that butyrate kinase (EC:2.7.2.7) is the finale  
299 enzyme to produce butyrate from another branch pathway. Therefore, butyryl-CoA  
300 directly produces butyrate via acetate-CoA transferase (EC: 2.8.3.8), rather than  
301 another branch pathway. This result may be due to the high content of rumen  
302 microbes that produce butyrate or use of acetate as a precursor for butyrate synthesis  
303 under conditions of lactate fermentation (where lactate fermentation could occur).

### 304 **3.5 The major microbial material involved in cellulose and volatile fatty acid** 305 **metabolism gene characterization and quantification**

306 The amount of major microbial material involved in cellulose and VFA metabolism  
307 genes was estimated from the relative abundance of rumen total bacteria, which was  
308 determined by qPCR using specific bacterial primers of HF and HC groups. In our  
309 study, we selected eight representative genes (Tab. 5). At present, *Ruminococcus albus*  
310 and *R. flavefaciens* are considered to be the major cellulolytic bacteria in the rumen  
311 (25), and the relative abundance in the HF group is higher than in the HC group.  
312 *Ruminococcus albus* and *R. flavefaciens* both have relative content at approximately  
313 7%. *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* are the main cellulolytic  
314 bacterial, and the relative content of the HF group was significantly higher ( $P \leq 0.05$ )  
315 compared to that of the HC group. These results confirmed the previous research in  
316 this study on cellulose and enzyme activity. The relative content of the species  
317 *Prevotella ruminicola*, *Selenomonas ruminantium*, and *Megasphaera elsdenii* were  
318 higher in the HC group than in the HF group.

## 319 **4、DISCUSSION**

### 320 **4.1 Rumen fermentation parameters and rumen contents**

321 In the present study, we investigated the fermentation parameters, Our findings show  
322 that HC diet produce more VFAs (Tab.2), and the accumulation of VFAs in the rumen  
323 resulted in a decrease in rumen pH (26). The concentration of NH<sub>3</sub>-N decreased  
324 significantly with the increase of concentrate in the diet. Michalski et al.(2014)(27)  
325 found that non-fibrous carbohydrates (monosaccharides, disaccharides,  
326 polysaccharides, etc.) in the diet are the main factors limiting the utilization of NH<sub>3</sub>-N

327 by rumen microbes and that raising the level of nonfibrous carbohydrates in the diet  
328 can promote utilization of NH<sub>3</sub>-N by rumen microbes.

329 In the rumen content, the HC group has a higher content of cellobiose and glucose  
330 compared to the HF group. Cellobiose generates glucose by  $\beta$ -glucosidase, Chen(2012)  
331 (28) showed that  $\beta$ -glucosidase is a key factor in the conversion of cellobiose to  
332 glucose and enhancing the efficiency of cellulolytic enzymes for glucose production.  
333 In this study, the cellulose digestibility of HC group was shown to be higher than for  
334 HF group may be due to the content of cellulose in the HC group was lower and the  
335 content of the cellobiose decomposed by cellulose and glucose decomposed by  
336 cellobiose were higher than the HF group.

337 Xylan is the main component of hemicellulose; therefore, the degradation of  
338 hemicellulose was studied with xylan changes. In our research, the  $\beta$ -xylosidase in the  
339 HC group was significantly higher than in the HF group.  $\beta$ -xylosidase is an  
340 exo-enzyme that mainly catalyzes the hydrolysis of xyloside and from the  
341 nonreducing hydrolysis xylo-oligosaccharide to xylose (29, 30), which can effectively  
342 relieve feedback inhibitions of xylanase by endo-xylanase hydrolysates  
343 (xylo-oligosaccharides) (31). Hemicellulose digestibility of HC group was shown to  
344 be higher than for HF group, which is consistent with the digestibility of cellulose.

#### 345 **4.2 Ruminal microbial community changes within HC and HF groups**

346 At the family level, The rumen microbial community comprises mainly  
347 *Acidaminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae* and  
348 *Succinivibrionaceae*, which accounted for approximately 65%. At the genus level, our  
349 study focused on the main and different microbial other than the all microbial.  
350 *Prevotella* (family *Prevotellaceae*), *Selenomonas\_1* and *Veillonella* (both from the  
351 family *Acidaminococcaceae*). *Prevotella*, as part of the core microbiome, can grow  
352 rapidly on starch media and produce final products other than lactate (mainly  
353 succinate and propionate) (32). The reason for the enrichment of *Succinivibrio* and  
354 *Selenomonas* in the HC group may be related to the diet in which the HC group has  
355 more starch and maltose and less cellulose and xylan. Previous researches (33-35)  
356 have shown that *Succinivibrio* and *Selenomonas* growth on starch, maltose and

357 soluble sugars and cellulose and xylan are not available, which produces more  
358 succinate, which decarboxylates and leads to more propionate formation (36).  
359 Recently, extensive investigations have been carried out on the microbial communities  
360 of multiple groups of cows with different F:C ratios. *Ruminococcus* (family  
361 *Lachnospiraceae*) were the principal rumen cellulose-degrading bacteria. Cerrillo et al.  
362 (1999) (37) reported that the forage group was abundant in *Ruminococcus*, among  
363 other cellulose-degradation bacteria, and studies have shown that *Ruminococcus* are  
364 fermented with a large amount of cellulose as a substrate and can form more acetate  
365 (38, 39), which is consistent with the results of our study of the forage group to  
366 produce more acetate and have more cellulose and hemicellulose.

### 367 **4.3 Volatile fatty acid metabolism**

368 Volatile fatty acids act as energy sources for ruminants; therefore, it was important to  
369 study the metabolism of VFAs. The production of acetate was contained in the  
370 metabolic pathway of butyrate; therefore, the metabolism of propionate and butyrate  
371 was the main research focus.

#### 372 **4.3.1 Genes directly involved in propionate**

373 In rumen, plant polysaccharides are fermented by the rumen microbial, propionate is  
374 one of the final production. Propionate can be produced by acrylate pathway and  
375 succinate pathway. The acrylate pathway is the primary pathway in the case of animal  
376 diets with high starch content (40, 41), and *Megasphaera elsdenii* is the major  
377 propionate producer via the acrylate pathway (42, 43), in our study, the content of  
378 *Megasphaera elsdenii* was very low at the expression (Tab.5) and genes for  
379 lactyl-CoA dehydratase was not found. Conversely, fermenting to propionate seems to  
380 be more likely given the high readings of the genes involved via the succinate  
381 pathway. The CO<sub>2</sub> was immobilized in phosphoenolpyruvate to form oxaloacetate,  
382 which was then succinate produced by malate and fumarate, and succinate is rapidly  
383 converted to propionate by the microbial enzyme (32). Such a pathway has been  
384 demonstrated in *Veillonella* (44) (Fig. 4). Studies have shown that *Bacteroides*  
385 *ruminicola* with glucose is the main fermentation substrate, and the main fermentation  
386 products are succinate, CO<sub>2</sub>, formate and acetate (45, 46). In vitro fermentation

387 studies have shown that succinate as a result of carbohydrate fermentation is rapidly  
388 converted to propionate in the rumen (32). *Selenomonas ruminantium*, another rumen  
389 species, can also produce propionate by fermenting carbohydrates or lactate, or  
390 produce propionate by decarboxylation of succinate. VFA produced by rumen  
391 fermentation can be used as an energy supply for animals, of which only propionate  
392 gluconeogenesis is the main source of glucose. Therefore, propionate has important  
393 physiological significance for ruminants.

394

#### 395 **4.3.2 Genes directly involved in butyrate**

396 The content of butyrate in HF group was higher than HC group, this result was  
397 consistent with McCullough and Sisk research. McCullough and Sisk (47) studied the  
398 effects of different diets on rumen VFAs and found that the butyrate yield in the  
399 forage group was higher than that in the grain group. This result may be due to the  
400 high relative content of rumen microbes that produce butyrate. Butyrate production  
401 and accumulation appear to increase when high-fiber degradability coincides with the  
402 high availability of nonstructural carbohydrates (48). This also may be related to the  
403 use of acetate as a precursor for butyrate synthesis under conditions of lactate  
404 fermentation (where lactate fermentation could occur). Under these conditions,  
405 lactate-producing bacteria such as *Butyrivibrio fibrisolvens* can directly produce  
406 butyrate using acetate via butyryl-CoA/acetic acid-CoA transferase (EC: 2.8.3.8)  
407 rather than by converting two acetyl-CoA molecules into acetoacetyl-CoA (49). The  
408 *Butyrivibrio fibrisolvens* relative content at the transcriptional level from our study  
409 was similar to the findings of Diezgonzalez et al. (1999), which was the HF group  
410 with the higher *Butyrivibrio fibrisolvens* relative content, and then the butyrate of the  
411 forage group was higher. On the other hand, *M. elsdenii* produces butyrate via the  
412 malonyl-CoA pathway from various reactions involving acetyl-CoA, which is  
413 activated by acetate and is combined with CO<sub>2</sub> to form malonyl-CoA (50). The lactate  
414 fermentation of *M. elsdenii* is not regulated by glucose or maltose, so the utilization of  
415 lactic acid increases with the feeding of soluble sugar (43, 51). Others have also  
416 reported that when *M. elsdenii* is purely cultured, the accumulation of butyrate

417 prevented high levels of lactate accumulation. The magnitude of the effect was  
418 positively correlated with the dose of *M. elsdenii*. Our recent work indicated that the  
419 relative content of *M. elsdenii* was very low in both HF and HC groups; however,  
420 under this condition, fermentation to butyrate seems more likely via the  
421 butyryl-CoA/acetate-CoA transferase pathway using acetate as an acceptor.

#### 422 **4.4 The major microbial material involved in cellulose and volatile fatty acid** 423 **metabolism gene characterization and quantification**

424 Abundance of microbial involved in cellulose and VFA metabolism encoding genes  
425 was measured by quantifying the microbial gene copy number as well as the  
426 expression level. Based on the DNA level, different patterns of abundance of  
427 microbial encoding genes were found in HF and HC groups. *Ruminococcus albus* and  
428 *R. flavefaciens* both have relative content at approximately 7%. Previous studies (17,  
429 25) have shown that they constitute up to approximately 10% of the total bacterial  
430 isolates in either HF or HC diets, and increasing rumen *Ruminococcus* increases the  
431 ratio of A:P. Our present study was consistent with Weimer(1998) (52).

432 The enzyme produced by the *Fibrobacter succinogenes* was confirmed to have an  
433 independent cellulose catalytic zone and a cellulose binding zone (53) and has a  
434 strong ability to degrade plant cell walls. The *Fibrobacter succinogenes* can produces  
435 a variety of  $\beta$ -glucanases, which degrade cellulose and xylanase, which in turn  
436 degrades hemicellulose. *Butyrivibrio fibrisolvens*, another rumen species, produces  
437 xylanase and endoglucanase (54). These results confirmed the previous research in  
438 this study on cellulose and enzyme activity. *Prevotella ruminicola* is a dominant  
439 bacterium in the rumen and specifically degrades the noncellulosic components in the  
440 plant cell wall and can simultaneously metabolize pentose and glucose but first  
441 utilizes pentose and then cellobiose (55). Therefore, in the rumen, we presumed that  
442 the pentose (main xylose) content is lower than the cellobiose content. Our  
443 observations confirm that the content of xylose (Fig.2) was lower than cellobiose in  
444 the rumen (Fig.1). *Selenomonas ruminantium* is unable degrade cellulose directly, but  
445 it can produce succinate by using the cellobiose of the cellulose degradation product,  
446 and *Selenomonas ruminantium* and *Veillonella alkalescens* via the succinate pathway

447 produce propionate (56). *Selenomonas ruminantium* can also produce acetate via the  
448 acetyl-CoA pathway, so it plays an important role in the metabolism of propionate. In  
449 the rumen, *Megasphaera elsdenii* is regarded as the main fermenter of lactate (51)  
450 under the conditions of rapid fermentation of sugar and production of more lactate,  
451 which results in the relative content in the bacterial community increasing (57). In our  
452 present study, the relative content of *Megasphaera elsdenii* in the HFgroup was higher  
453 than that in the HC group, and coincident with that, the lactate content was also higher  
454 in the HF group, However, the HC group should contain more sugar, and the relative  
455 content of *Megasphaera elsdenii* was higher, which may be related to our sampling  
456 time and the interaction between microorganisms, which requires further research.

## 457 **5、 CONCLUSION**

458 In conclusion, this study combined metagenomics and metabolism to explore the  
459 effects of HF and HC diets on the cellulose degradation process and ruminal microbial  
460 communities in rumen. Feeding a HF diet increased ruminal pH and decreased TVFA  
461 concentration. The content of *Ruminococcus*, *Papillibacter* and *Roseburia* in HF  
462 group were higher, which could efficiently degrade cellulose in rumen, thereby  
463 enhancing the activity of CMCase by promoting microorganisms producing this  
464 enzyme. We discover that the butyryl-CoA dehydrogenase (EC:1.3.8.1) is the  
465 restriction enzyme in butyrate metabolism and succinate-CoA synthetase (EC:6.2.1.5)  
466 and lactyl transferase (EC:2.8.3.1) are the key enzyme in pyruvate metabolism, can  
467 reveal how fiber degradation and VFAs production are manipulated by metabolic  
468 pathways and microbial communities. Therefore, combined with cellulose, enzymes  
469 and VFA measurements, rumen microbiome and fermentate characterization will be a  
470 useful screening tool for choosing cellulolytic bacteria.

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**Tab. 1 Primers used for real-time PCR quantification**

Target bacteria	Primer	T <sub>m</sub> (°C)	Product size(bp)	Reference
General Bacteria 16Sr DNA	F: 5'-CGGCAACGAGCGCAACCC -3' R: 5'-CCATTGTAGCACGTGTGTAGCC -3'	58	130	(23)
<i>Ruminococcus albus</i>	F: 5'-CCCTAAAAGCAGTCTTAGTTCG-3' R: 5'-CCTCCTTGCGGTTAGAACA-3'	54	176	(24)
<i>Ruminococcus flavefaciens</i>	F: 5'-CGAACGGAGATAATTTGAGTTTACTTAGG-3' R: 5'-CGGTCTCTGTATGTTATGAGGTATTACC-3'	58	132	(24)
<i>Fibrobacter succinogenes</i>	F: 5'-GGAGCGTAGGCGGAGATTCA-3' R: 5'-GCCTGCCCTGAACTATCCA-3'	59	97	(24)
<i>Butyrivibrio fibrisolvens</i>	F: 5'-ACCGCATAAGCGCACGGA-3' R: 5'-CGGGTCCATCTTGTACCGATAAAT-3'	59	124	(24)
<i>Prevotella ruminicola</i>	R: 5'-GCGAAAGTCGGATTAATGCTCTATG-3' F: 5'-CCCATCCTATAGCGGTAAACCTTTG-3'	59	78	(24)
<i>Selenomonas ruminantium</i>	R: 5'-GGCGGGAAGGCAAGTCAGTC-3' F: 5'-CCTCTCCTGCACTCAAGAAAGACAG-3'	60	83	(24)
<i>Megasphaera elsdenii</i>	R: 5'-GACCGAAACTGCGATGCTAGA-3' F: 5'-CGCCTCAGCGTCAGTTGTC-3'	58	129	(24)
<i>Veillonella alkalescens</i>	GACGAAAGTCTGACGGAGCA TGCCACCTACGTATTACCGC	60	132	This study

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**Tab. 2 Fermentation products of carbohydrates in the rumen**

	Con	Forage	SEM	P-value
pH	5.76	6.14	0.0719	0.0208
NH <sub>3</sub> -N, mg/dL	11.94	16.51	0.5363	<.0001
Acetate, mM	86.41	80.09	2.1322	0.5929
Propionate, mM	41.82	23.52	1.0549	<.0001
Butyrate, mM	16.89	12.96	0.4438	0.0002
Valerate, mM	2.77	0.67	0.0234	<.0001

Isobutyrate, mM	1.24	4.17	0.1179	<.0001
Isovalerate, mM	1.92	0.78	0.0537	<.0001
TVFA, mM	151.04	130.18	3.5658	0.0033
Acetate: Propionate	2.07	3.76	0.0711	<.0001
Lactate (mmol/L)	6.61	8.48	0.48	0.0213

666 <sup>a</sup>TVFA: Total volatile fatty acid

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**Tab. 3 The chemical composition of diets and rumen contents**

	Con	Forage	SEM	P-value
Ruminal contents				
Cellulose	22.07	23.13	2.14	0.8013
Hemicellulose	22.83	31.88	7.22	0.5442
Acid detergent lignin (ADL)	12.24	17.93	1.96	0.1616
Diets				
Cellulose	13.16	16.69	0.25	<.0001
Hemicellulose	16.02	28.88	1.25	<.0001
Acid detergent lignin	6.00	8.61	0.20	<.0001

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675 **Tab. 4 Bacterial taxa (97% sequence similarity) with taxonomy assigned to the highest**  
 676 **possible resolution, differing in mean relative abundance (%) between the HF and HC**  
 677 **groups measured.**

Taxon: order/family/genus	F:C diet	16S rDNA			Metagenome		
		F	Con.	P	F	Con.	P
Clostridia/Lachnospiraceae/Ruminococcus	F	2.01	0.08	0.124	1.94	0.07	0.003
Ruminococcaceae /Ruminococcaceae_UCG-010	F	1.54	0.09	<.001	1.37	0.09	0.001
Clostridia/Acidaminococcaceae/ Papillibacter	F	1.48	0.08	<.001	1.40	0.10	0.002
Clostridia/Lachnospiraceae/ Roseburia	F	1.54	0.19	0.001	1.61	0.18	<.001
Bacteroidetes/Prevotellaceae/ Prevotella	Con.	0.24	3.44	0.005	0.21	3.47	0.003
Clostridia/Acidaminococcaceae /Veillonella	Con.	0.23	1.22	0.008	0.20	1.43	0.002
Gammaproteobacteria/ Succinivibrionaceae	Con.	0.12	2.25	<.001	0.11	2.15	<.001
/Succinivibrio							
Clostridia/Acidaminococcaceae/Selenomonas_1	Con.	0.33	2.53	0.026	0.30	2.42	0.004

678 Taxa with significant differences ( $P \leq 0.05$ ) in either 16S rRNA gene amplicon or 16S rRNA gene metagenome  
 679 sequence abundance are shown. Significances are based on GLM and Student's t-test corrected P values. NS: not  
 680 significant; NA: not applicable

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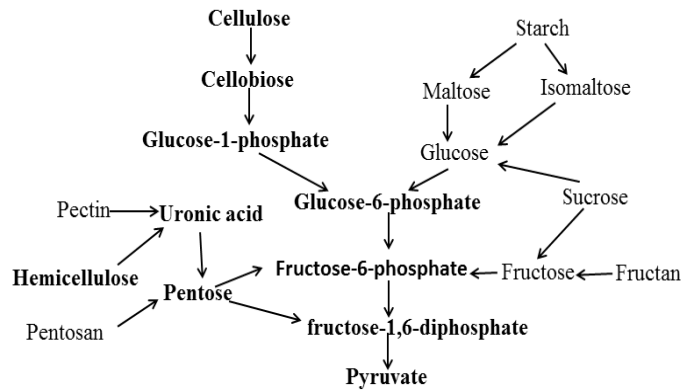
694 **Tab. 5 Differences in the relative expression (%) of the main bacteria in the HF and HC groups.**

695 The relative expression (%) levels are shown.

Main microbial	Forage group	Concentrate group	SEM	<i>p</i> -value
<i>Ruminococcus flavefaciens</i>	5.0003	3.8988	0.4994	0.1499
<i>Ruminococcus albus</i>	2.2981	2.7131	0.4303	0.5207
<i>Fibrobacter succinogenes</i>	0.4214	0.3268	0.0691	0.0032
<i>Butyrivibrio fibrisolvens</i>	0.0486	0.0201	0.0065	0.0112
<i>Prevotella ruminicola</i>	2.7294	4.3986	0.3606	0.0113
<i>Selenomonas ruminantium</i>	1.6891	3.7616	0.2054	0.0022
<i>Megasphaera elsdenii</i>	0.0191	0.0034	0.0008	0.0001
<i>Veillonella alcalescens</i>	0.0339	0.2068	0.0320	0.0088

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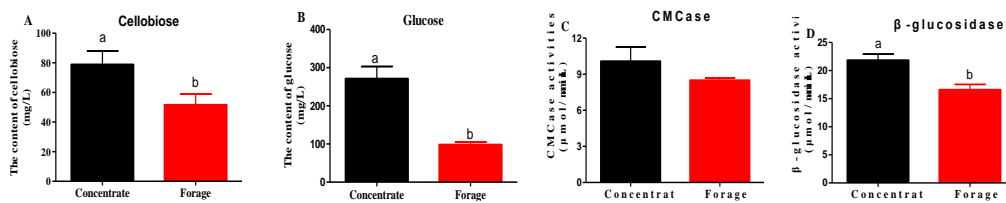
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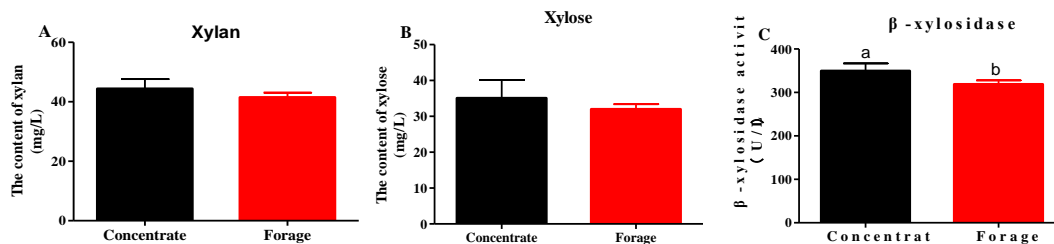
722 **Fig.1 Degradation of polysaccharides to produce pyruvate (6)**

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728 Fig. 2 Cellulose decomposition of rumen contents. (A) Cellobiose, (B) Glucose, (C) CMCCase, (D)  
729 β-glucosidase. The error bars represent the standard error of the mean (n = 3). Different letters in each  
730 figure panel indicate a significant difference (P < 0.05).



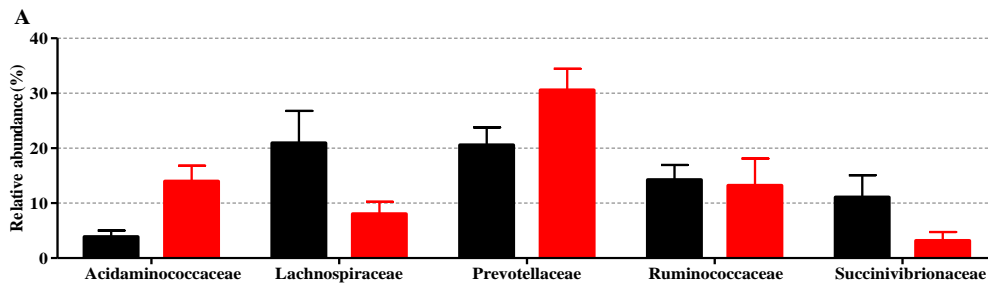
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732 Fig. 3 Hemicellulose decomposition of rumen content. (A) Xylan, (B) Xylose, (C) β-xylosidase. The  
733 error bars represent a standard error of the mean (n = 3). Different letters in each figure panel indicate a  
734 significant difference (P < 0.05).

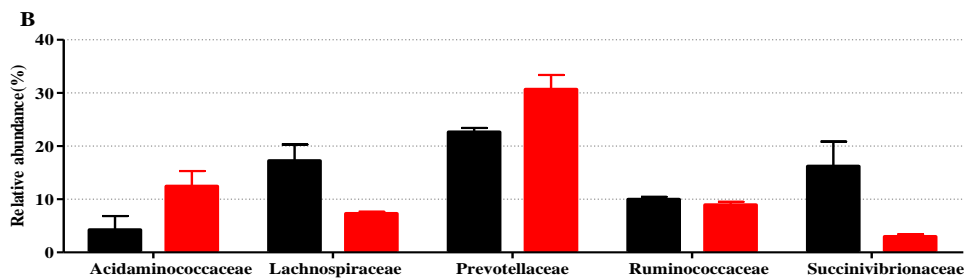
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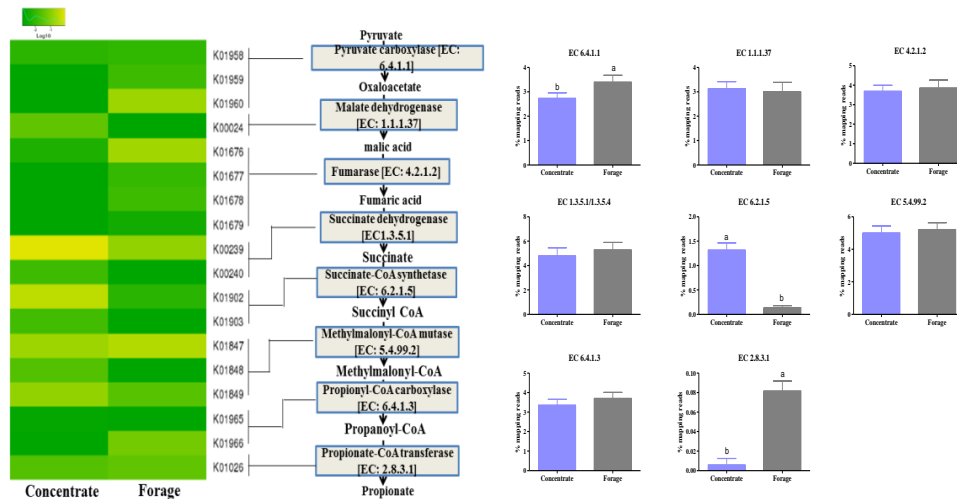
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744 Fig. 4 Relative abundance of the most highly represented bacterial families based on 16S rDNA gene amplicon  
 745 sequencing data (a) and 16S rRNA genes retrieved from the metagenome dataset (b) from rumen content samples  
 746 of the HF group (black) and HC group (red). \*\*P < 0.01, \*P < 0.05. Error bars denote one standard deviation.

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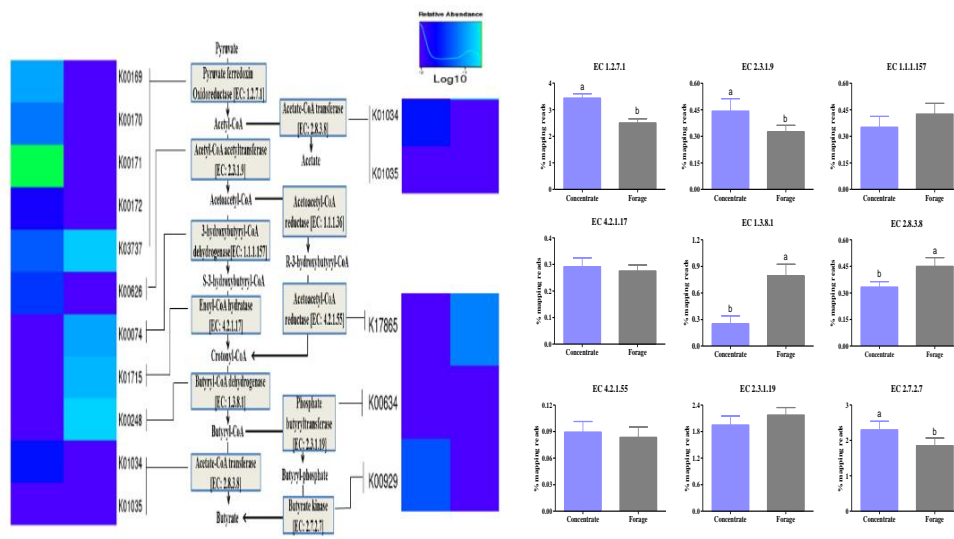
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751 Fig. 5 The metagenomic abundance of key elements of the propionate production pathway. Center  
 752 pane: the propionate production pathway showing enzyme classification (EC) numbers. Left pane:  
 753 heat map of KEGG orthologues for the EC numbers involved in propionate production (lines  
 754 connect the heat map to the propionate production pathway indicating which K0 numbers  
 755 represent the given enzymes). Right pane: the abundance of each of the relevant EC numbers in  
 756 our data set. The bar charts show the percentage of reads mapped to each enzyme in the 2 groups  
 757 (HC and HF groups). The blue bars are cattle selected for HC groups, and gray bars are cattle  
 758 selected for HF groups.

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763 Fig. 6 The metagenomic abundance of key elements of the butyrate production pathway. Centre  
764 pane: the butyrate production pathway, plus ancillary reactions, showing enzyme classification  
765 (EC) numbers. Left and right pane: heat map of KEGG orthologues for the EC numbers involved  
766 in butyrate production (lines connect the heat map to the butyrate production pathway, indicating  
767 which K0 numbers represent the given enzymes). Lower pane: the abundance of each of the  
768 relevant EC numbers in our data set. Bar charts show the percentage of reads mapped to each  
769 enzyme in the 2 groups for diet (high or low concentrate). Grey bars are cattle selected for HF  
770 groups and blue bars are cattle selected for HC groups.

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