Exploring the Diversity of Active Ureolytic Bacteria in the Rumen by

Comparison of cDNA and gDNA

Sijia Liu, Nan Zheng, Shengguo Zhao*[1], Jiaqi Wang

Abstract

Background: Ureolytic bacteria produce urease that hydrolyzes dietary or recycled urea to ammonia, which can conversion into microbial proteins which are ideal precursor for lactoprotein. The diversity of ruminal ureolytic bacteria benefits N utilization efficiency in ruminants. The urease gene (*ureC*) has been used as a marker to characterize the diversity of ruminal ureolytic bacteria at the genomic DNA (gDNA) level. However, there is no information at the complementary DNA (cDNA) level to reflect the active status of ureolytic bacteria. To reveal the diversity of active ureolytic bacteria in the rumen, we compared *ureC* amplicons between gDNA and cDNA.

Results: The sampling time had no significant difference on the alpha and beta diversity indices of the ureolytic bacterial. The Shannon diversity of the *ureC* gene for cDNA was greater than that for gDNA ($p < 0.05$). There were significant difference in the beta diversity of ureolytic bacterial between gDNA and cDNA ($p < 0.01$), which indicates a shift in the community of active ureolytic bacteria. Approximately 67% of *ureC* sequences from cDNA could not be confidently classified at the genus level. The active ureolytic bacteria were mainly from *Helicobacter*, *Herbaspirillum*, *Clostridium*, *Paenibacillus*, *Synechococcus*, and *Sphingobacterium* sp. Changes in the operational taxonomic units revealed that the top abundant *ureC* genes were mostly consistent

Corresponding author: Shengguo Zhao；e-mail: zhaoshengguo1984@163.com; Tel:010-62816069
State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, No.2 Yuanmingyuan West Road, Haidian, Beijing, P. R. China

22   between gDNA and cDNA, and most differences occurred in the *ureC* genes with

23   lower abundances.

24   **Conclusion:** These results revealed distinct ureolytic bacterial community profiles

25   based on gDNA and cDNA. The dominant ureolytic bacterial had high transcriptional

26   activity, and the differential were mainly distributed in the genus of low abundance.

27   **Keywords:** rumen, ureolytic bacteria, active, diversity

Background

Urea is an economical non-protein of nitrogen in feed for ruminants[1]. Ureolytic bacteria produce ureases in the rumen, which is a key enzyme in the hydrolysis of dietary urea to ammonia and carbon dioxide[2]. Ammonia derived from urea can conversion into microbial proteins, which are ideal to promote animal growth and production[3]. Excess ammonia is synthesized endogenous urea in the liver that is then recycled via the ruminal wall and salivary secretion[4]. Urea recycling is an important biological process in the rumen in response to low dietary nitrogen[5]. However, hyperactivity of urease activity produces excess ammonia which is excreted in the urine which reduced the quality of lactoprotein and pollutes the environment. Therefore, elucidation of diversity of rumen ureolytic bacteria is important to increase the efficiency of urea-N utilization for ruminants.

We assessed the diversity of ureolytic bacteria in the rumen, based on analysis of gDNA[6]. Although gDNA-based techniques have provided important insights, there persist shortcomings in the ability to identify active microbes in the rumen[7]. The detected gDNA may originate from dead or inactive bacteria[8]. A large number of *ureC* genes have been identified in the rumen, but the expression patterns of these genes remain unclear. In general, RNA is reportedly a more reliable indicator of bacterial viability than target genes of gDNA[9-11]. Qi et al.[12] examined fungal gene expression in the rumen of muskoxen (*Ovibos* moschatus) by analysis of a library of cDNA.

49 Sequencing of cDNA amplicons from reverse-transcription of RNA is better suited to

50 assess the *in situ* activity of the microbial community because the concentration of

51 RNA is generally well-correlated to the growth rate and activity[13]. Under stress,

52 endonuclease can initiate degradation of functional ribosomes, whereas homologs in

53 physically damaged or dying bacteria can be degraded by homologs of ribonuclease

54 I[14]. The bacterial ribosomes relatively labile characteristic has been used in

55 numerous studies to better assess the active and viable components of microbial

56 communities[14, 15].

57 To investigate the diversity of active ureolytic bacteria, we compared *ureC* amplicons

58 between gDNA and cDNA in the rumen. This survey is expected to expanded current

59 knowledge of the active ureolytic microbial community in the rumen in order to

60 provide a basis for the design of regulation rumen ureolytic bacteria composition to

61 increase the efficiency of urea-N utilization for ruminants.

62 **Methods**

63 **Animals and sampling**

64 Rumen fluid samples were collected from four non-lactating Holstein dairy cows

65 (body weight, 550 ± 50 kg) fitted with ruminal fistulas. The dairy cows were owned

66 by the Animal Care and Use Committee for Livestock of the Institute of Animal

67 Sciences, Chinese Academy of Agricultural Sciences. Diet of cows were total mixed

68 ration, which consisted of 36% corn silage, 16% corn, 14.6% syrup vinasse, 5.9%

69 soybean meal, 5.7% dried distillers grains with solubles, 5.4% soybean hulls, 5.3%

70 barley, 5.0% oat grass, 4.0% alfalfa, 0.8%, $CaHPO_4$, 0.5% $NaHCO_3$, 0.2% NaCl, 0.2%

71 $CaCO_3$, 0.1%, $C_5H_{14}ClNO$, 0.1% calcium fatty acid, 0.1% double beneficial element,

72 and 0.1% rhodamine. Samples of the rumen contents of each cow were obtained at 0,

73 2 and 6 h after morning feeding. The rumen fluid samples were stored in liquid

74 nitrogen prior to analysis.

75 **gDNA extraction and cDNA reverse transcription**

76 Total gDNA was extracted using the cetyltrimethylammonium bromide method, as

77 described previously[16]. Total RNA was extracted using TRIzol method. Rumen

78 fluid samples were homogenized for 5 min with steel balls (one with a diameter of 20

79 mm and 10 with diameters of 5 mm) into fine powder in liquid nitrogen using a

80 CryoMill (Retsch GmbH). Then, 3 g of the rumen fluid samples were incubated in 15

81 mL of TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) at room

82 temperature for 5 min. Afterward, 4 mL of chloroform were added to the samples by

83 vortexing for 15 s and then each was incubated at room temperature for 3 min.

84 Following centrifugation at $13,000 \times g$ for 15 min at 4ºC, the aqueous phase was

85 transferred to a fresh tube. Following the addition of 10 mL of isopropyl alcohol, the

86 tubes were vortexed for 30 s and then incubated at room temperature for 10 min.

87 Afterward, the samples were centrifuged at $13,000 \times g$ for 10 min at 4ºC and the

88 supernatant was discarded. The RNA pellet was washed once with 75% ethanol and

89 resuspended in 10 mL of 75% ethanol by hand mixing and then centrifuged at $7500 \times$

90 g for 10 min at 4ºC. Once the supernatant was removed, the RNA pellet was air-dried

91 and dissolved in RNase-free water by passing the solution a few times through a

92 pipette tip, and then incubated for 10 min at 55ºC. Trace gDNA in the RNA samples

93 was removed by incubation with 4 µL of DNase I (Takara Bio, Inc., Kusatsu, Shiga

94 Prefecture, Japan) per 100 µg of total RNA for 30 min at 37°C. Then, the RNA was

95 further purified using an RNAclean Kit (Tiangen Biotech Co., Ltd., Beijing, China).

96 The 16S rRNA gene was amplified using the primers 27F (5´-GAG TTT GAT CCT

97 GGC TCA G-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´). Purified RNA

98 was used to check whether the purified RNA samples contained residual gDNA. The

99 integrity and concentration of the total RNA were assessed using an Agilent 2100

100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). RNA samples with a

101 RNA integrity number > 8.0 were deemed suitable for reverse transcription of cDNA,

102 which was synthesized using FastQuant RT Super Mix (Tiangen Biotech Co., Ltd.)

103 and random primers in accordance with established procedures.

104 **PCR amplification and sequencing of *ureC* genes**

105 *UreC* genes were amplified with the primer set *UreC*-F (5´-TGG GCC TTA AAA

106 THC AYG ARG AYT GGG-3´) and *UreC*-R (5´-GGT GGT GGC ACA CCA TNA

107 NCA TRTC-3´)[17]. Reactions were performed in a MyCycler Thermal Cycler

108 (Bio-Rad, USA) using a 50-µL mixture containing 5 µL of 10 × PCR buffer

109 (Invitrogen Corporation), 1 µL of dNTP mixture (10 mM), 1.5 µL of each forward

110 and reverse primer (10 µM), 0.25 µL of Platinum Taq DNA polymerase (Invitrogen

111 Corporation), 2 µL of gDNA or cDNA (100 ng/µL), and 38.75 µL of sterile

112 double-distilled H_2O. The PCR amplification consisted of denaturation at 94ºC for 5

113 min, followed by 30 cycles at 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 30 s, and a

114 final extension at 72ºC for 15 min[6]. PCR amplicons were extracted from agarose

115 gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Scientific Inc.,

116 Union City, CA, USA) in accordance with the manufacturer's instructions and

117 quantified using the Qubit^{TM} Assay kit (Thermo Fisher Scientific). Purified amplicons

118 were pooled in equimolar amounts and paired ends were sequenced (2×300 bp) on

119 an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) by Shanghai

120 Majorbio Bio-pharm Technology Co., Ltd. (Shanghai. China).

121 **Statistic analysis**

122 Sequence analysis used methods described previously [6]. Using QIIME calculated

123 alpha and beta diversity indices and significant fold-changes of OTUs. Heatmaps of

124 the top 50 significantly different OTUs at the genus level were generated with

125 MicrobiomeAnalyst [18].

126 The differences between gDNA and cDNA for the top 20 *ureC* gene OTUs and top 10

127 ureolytic bacteria genera were analyzed using the paired Mann–Whitney test with

128 GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

129 Differences in beta diversity between gDNA and cDNA were determined by analysis

130 of similarity. The top 50 significant different ureC gene OTUs was identified using

131 the paired Mann–Whitney test with the MicrobiomeAnalyst web-based tool. A

132 probability (*p*) value of < 0.05 was considered statistically significant.

133 **Results**

134 **Alpha Diversity of *ureC* genes**

135   In total, 321,612 quality sequence reads were obtained. The average number of reads

136   was 14,619. The total sequences were assigned to 676 OTUs at a sequence similarity

137   cut-off of 97%.

138   Based on the *ureC* genes derived from gDNA and cDNA samples from different time

139   points, there were no significant differences in the Observed_species, Chao1, and

140   Shannon diversity indices (Additional file 1). The results indicated that the sampling

141   time had no significant difference on the alpha diversity of gDNA and cDNA. The

142   cDNA had higher evenness at 0 h, while the abundance significantly decreased after 2

143   h and the observed species significantly increased after 6 h (Additional file 2).

144   For the gDNA and cDNA of all the samples from different sampling times, there were

145   no significant differences ($p > 0.05$) in Observed_species (Figure 1 A) and Chao1

146   (Figure. 1 B). The Shannon index (Figure. 1 C) of the cDNA was greater than that of

147   the gDNA ($p < 0.05$). The *ureC* alpha diversity of the cDNA was significantly higher

148   than that of the gDNA.

149   **Beta diversity of *ureC* genes**

150   The ureolytic bacteria composition assessed by beta diversity. The sampling time had

151   no significant difference on the beta diversity of gDNA and cDNA ($p > 0.05$) (Figure.

152   2 A). However, there were significant differences in the beta diversities of gDNA and

153   cDNA of all samples at different sampling times ($p < 0.01$) (Figure. 2 B). There were

154   also significant differences in the compositions of active and total ureolytic bacteria.

155   **Composition of ureolytic bacteria**

8

156   Approximately 43% and 50% of the total cDNA and gDNA sequences, respectively,

157   could not be matched any known classification at phylum level. Approximately 67%

158   and 69% of the cDNA and gDNA sequences, respectively,   could not be confidently

159   classified at the genus level, while the remaining sequences were assigned to

160   *Helicobacter* (16% and 17%), *Herbaspirillum* (7% and 9%), *Clostridium* (2% and

161   2%), *Paenibacillus* (1% and 1%), *Synechococcus* (1% and 1%) and *Sphingobacterium*

162   (1% and 1%) (Fig. 3). Also, there were no significant differences between gDNA and

163   cDNA of the predominant ureolytic bacteria.

164   **Changes in the abundances of OTUs of the *ureC* genes**

165   Among the top 25 abundant OTUs of *ureC* genes, 18 (72%) were unclassified

166   ureolytic bacteria at the genus level. The others were mainly distributed in

167   *Helicobacter* (OTU0, 9, 27), *Herbaspirillum* (OTU16, 6, 11), and *Acinetobacter*

168   (OTU29). Among the top 25 OTUs with high abundances of gDNA and cDNA (Fig 4),

169   there were significant differences in a cluster of OTUs (6, 27, 13, 15, 11, 1750, and

170   17). Those OTUs with high abundances of gDNA did not necessarily have high

171   abundances of cDNA. The top 50 OTUs with significant differences in abundances in

172   gDNA and cDNA were identified (Fig. 5). Changes to the OTUs revealed that the

173   most abundant *ure*C genes were similar between gDNA and cDNA, and most

174   differences were observed in diverse *ureC* genes with low abundances.

175   **Discussion**

176 It has been reported that RNA levels are directly related to the synthesis potential and

177 activity of microbial proteins[19]·[20]. Therefore, the data obtained from cDNA

178 sequencing could potentially be used as an index to taxonomically assess potentially

179 active microbes. Li et al. sequenced targeted RNA and gDNA amplicons to identify

180 and quantify potentially active rumen microbiota, and found significant differences in

181 the taxonomic classifications and community structures[21]. However, sequencing of

182 ureolytic bacteria based on gDNA may be misleading in regard to expressed or active

183 communities. In the present study, quantification of both gDNA and cDNA allowed

184 assessment of the differences in the abundances of major expressed or active and total

185 ureolytic bacteria and the community structure in the rumen.

186 In support of this view, the Shannon diversity of cDNA was greater than that of

187 gDNA, sampled at 0 h, indicating that a high community evenness of active bacteria.

188 The Chao1 index of gDNA was greater than that of cDNA after feeding for 2 h,

189 indicating that the total community abundance was higher, which may be due to the

190 crude protein content in the basal diet to provide adequate amounts of ammonia,

191 amino acids, and/or peptides for the synthesis of microbial proteins[22, 23]. However,

192 bacteria can utilize organic forms of nitrogen for the synthesis of microbial

193 proteins[24]. For samples collected at 6 h after feeding, the Observed_species of

194 cDNA was significantly greater than that based on gDNA, indicating that the

195 community richness of active taxa was much greater, possibly because the amount of

196 crude protein was insufficient, which resulted in the bacteria using urea nitrogen for

197 protein synthesis.

198 The beta diversity index of the gDNA was significantly greater than that of the cDNA.

199 In the process of urea decomposition, the abundance of active communities was

200 relatively lower, indicating that the expressed or active ureolytic bacteria communities

201 are more concentrated and specific, as not all *ureC* genes coded by gDNA are

202 expressed and actually function. The difference between gDNA and cDNA showed

203 that the active ureolytic bacteria communities was not associated with the total

204 community data[25]. cDNA is probably more indicative of active communities than

205 gDNA, and the dissimilarity in the diversity profiles of each emphasizes that the

206 results from gDNA should be interpreted cautiously with respect to inferences about

207 how communities actively respond to dynamic microenvironments.

208 Bacterial RNA has a short half-life and is very unstable, the cDNA are active at the

209 time of sampling[26], so the effect was assessed at different time points. The results

210 showed that the sampling time point had no significant impact on the alpha and beta

211 diversity indices of gDNA and cDNA, probably because the ureolytic bacteria were

212 functionally specific.

213 The *ureC* gene was used for analysis the ureolytic bacteria. The results showed that

214 the *ureC* gene OTUs were predominately from unclassified taxa. More than 50% and

215 43% of the *ureC* sequences of gDNA and cDNA, respectively, were not affiliated with

216 any known *ureC* genes at the phylum level, indicating that the rumen contained *ureC*

217 genes from unknown sources. Furthermore, most research on urease has been

218 conducted with the use of samples of soils[27-29] and ocean water[30-32]. Therefore,

219 the reference dataset used for taxonomic assignment of ureolytic bacteria in the rumen

220 should updated.

221 Changes to the top abundant ureolytic bacteria at the genus level were similar

222 between gDNA and cDNA. The highest proportion of classified u*reC* sequences were

223 from *Helicobacter* sp. Among the predominant OTUs, OTU0 was dominant in cDNA,

224 which was affiliated with *Helicobacter* sp. Hence, a previous study by our group[6]

225 investigated the predominant ureolytic bacteria in the rumen based on gDNA, which

226 revealed a high abundance of *Helicobacter* sp. in the rumen contents. By cloning and

227 sequencing the *ureC* gene, Zhao et al.[33] detected *ureC* diversity in the rumen and

228 found that 22% of the sequences were affiliated with *Helicobacter* sp. Coldham et

229 al.[34] isolated *Helicobacter* sp. from the gastrointestinal tract of sheep that tested

230 positive for urease activity. These findings of these studies are consistent with those of

231 the present study, which indicated that *Helicobacter* sp. are the major ureolytic

232 bacteria in the rumen, as determined by the high expression and activity levels. The

233 abundance of *Herbaspirillum* sp. was also relatively high in the rumen, although

234 urease activity was not investigated. *Herbaspirillum* sp. are rhizobacteria that promote

235 plant growth via the ability on fix nitrogen[35]. Diets of cows contain soybeans and

236 silage, which may contain *Herbaspirillum* sp. that are transplanted into the rumen.

237 However, further studies are needed to elucidate the mechanism underlying urea

238 usage in the rumen by *Herbaspirillum* sp. Also, *ureC* genes affiliated with

239 *Clostridium*, *Paenibacillus* and *Synechococcus* sp. were identified. *Clostridium* sp.

240 reportedly have urease activity[36]. A study conducted by Crociani et al.[37] of urease

activity in the stomach (fundus and antrum), caecal content, and soft rabbit feces showed that *Clostridium* sp. also had urease activity. Shi et al.[38] reported that *Paenibacillus polymyxa* strain NSY50 had the ability to increase urease activity by up to 2.25-fold. Jackie et al.[39] cloned and sequenced *Synechococcus sp.* strain WH7805 and found that the WH7805 urease had a predicted subunit composition typical of other bacterial ureases, although the organization of the WH7805 urease genes was unique. The results of these studies were consistent with those of the present study, which detected *ureC* genes of *Sphingobacterium* sp. However, Pinnaka et al.[40] isolated *Sphingobacterium bovisgrunnientis* strain YK2T from yak milk that had no urease activity, likely because the produced urease is active only in the rumen.

This is the first study to compare the ureolytic bacterial composition in the rumen between cDNA and gDNA datasets. The results illustrate differences between active (cDNA) and total (gDNA) ureolytic bacterial communities to describe the composition and characteristics of ureolytic bacterial active in the rumen. A single method cannot fully reveal the composition and diversity of ureolytic bacteria in the rumen. So, the accuracy of the data should be carefully considered in future studies. Difference in total (gDNA) and active (cDNA) ureolytic bacterial communities should be taken into account to better understand the structure and dynamics of ureolytic bacteria in the complex rumen ecosystem. Since the rumen harbors a large diversity of unclassified ureolytic bacteria, future studies are warranted to elucidate the mechanisms controlling urease synthesis.

Conclusion

In the present study, approximately 69% and 67% of the gDNA and cDNA sequences, respectively, of the ureolytic bacterial community in the rumen of dairy cows could not be confidently classified at the genus level. The sampling time had no significant difference on the alpha and beta diversity indices of gDNA and cDNA. However, the composition of active and total ureolytic bacterial communities were differences in the rumen. Moreover, there were no significant differences between gDNA and cDNA profiles of the most abundant ureolytic bacteria, as most differences were observed in *ureC* genes with the lowest abundance. The results contribute new data to ureolytic bacterial information in the rumen.

Abbreviations

ureC: urease gene

gDNA: genomic DNA

cDNA: complementary DNA

PCR: Polymerase Chain Reaction

dNTP: deoxy-ribonucleoside triphosphate

OTU: Optical Transform Unit

Declarations

Ethics approval and consent to participate

282 The experimental procedures involving the care and management of dairy cows were

283 approved by the Animal Care and Use Committee for Livestock of the Institute of

284 Animal Sciences, Chinese Academy of Agricultural Sciences (No. IAS201914).

285 **Consent for publication**

286 Not applicable

287 **Availability of data and materials**

288 All the sequences were submitted to the NCBI Sequence Read Archive

289 (SRA;http://www.ncbi.nlm.nih.gov/Traces/sra/), under accession number SRA:

290 SRP278349.

291 **Competing interests**

292 The authors have no conflict of interest to declare.

301 **Authors' contributions**

302   S.J.L and S.G.Z conceived the study and conducted the experimental

303   investigation. S.J.L and S.G.Z interpreted data and wrote the original draft

304   of the manuscript. J.Q.W and N.Z provided some materials for this study,

305   reviewed the manuscript and provided valuable suggestions. All authors

306   commented on the manuscript and approved the final manuscript.

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Figures
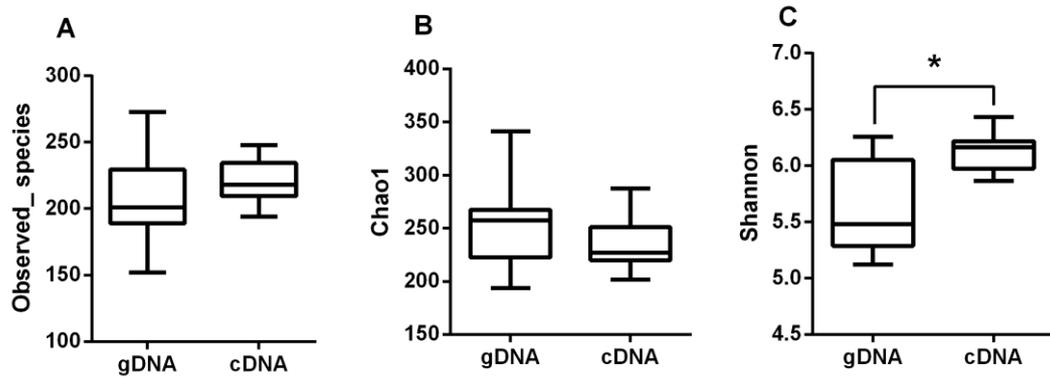
Fig. 1 Alpha diversity of rumen ureolytic bacterias of gDNA and cDNA. (A) Total observed species (B) Chao1 and, (C) Shannon index. Boxplots indicate the first and third quartiles with the median value indicated as a horizontal line the whickers extend to 1.5 times the inter quartile range. $*P < 0.05$.

Fig. 2 Beta diversity of rumen ureolytic bacterias of gDNA and cDNA. (A) Different sampling time, red dot represent 0 h, blue dot represent 2 h, green dot represent 6 h. (B) Ureolytic bacterias of gDNA and cDNA, red represent gDNA; green represent cDNA. Principle coordinate analysis (PCA) comparing changes in ureolytic bacterias based on weighted Unifrac distances.

Fig. 3 Taxonomic of the ureolytic bacteria components of gDNA and cDNA. The top 7 genera based on relative abundance are presented, the remaining genera grouped as "others".

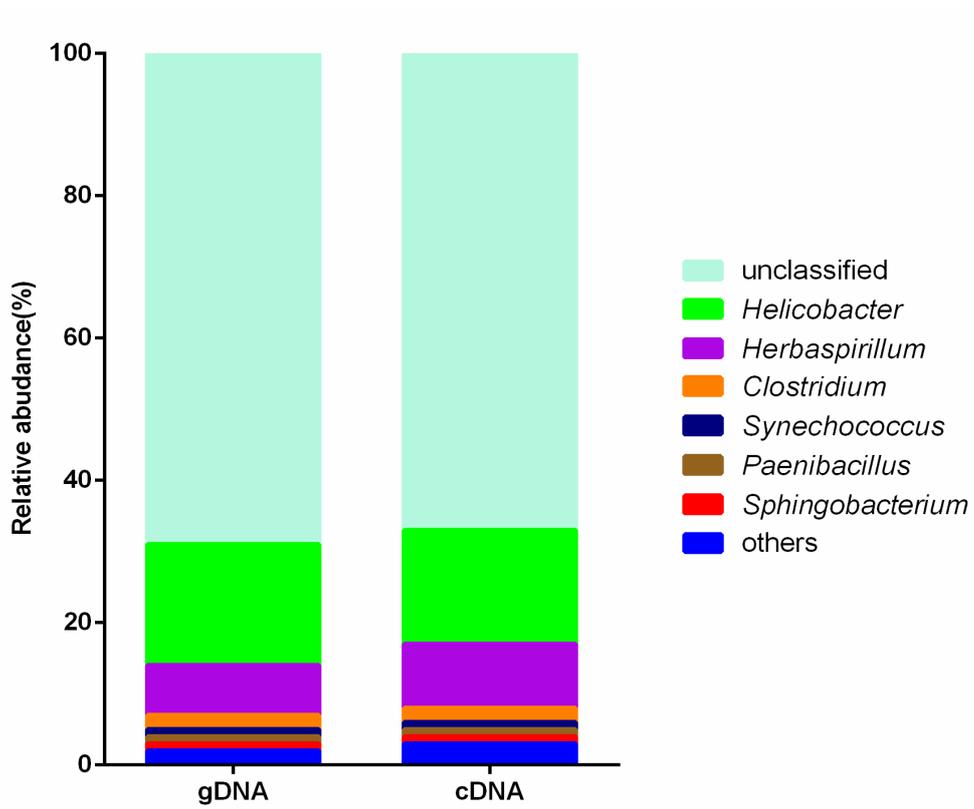**Fig. 4 The relative abundance of top 20 *ureC* gene OTUs.** In brackets represent bacteria genus. $*P < 0.05$..

Fig. 5 Heatmap of the top 50 significant difference *ureC* gene genus. Taxonomic assignment shows the genus level for each row. Color cell represent the relative abundance for a given genus level. Number represent OUT number, In brackets represent bacteria genus.
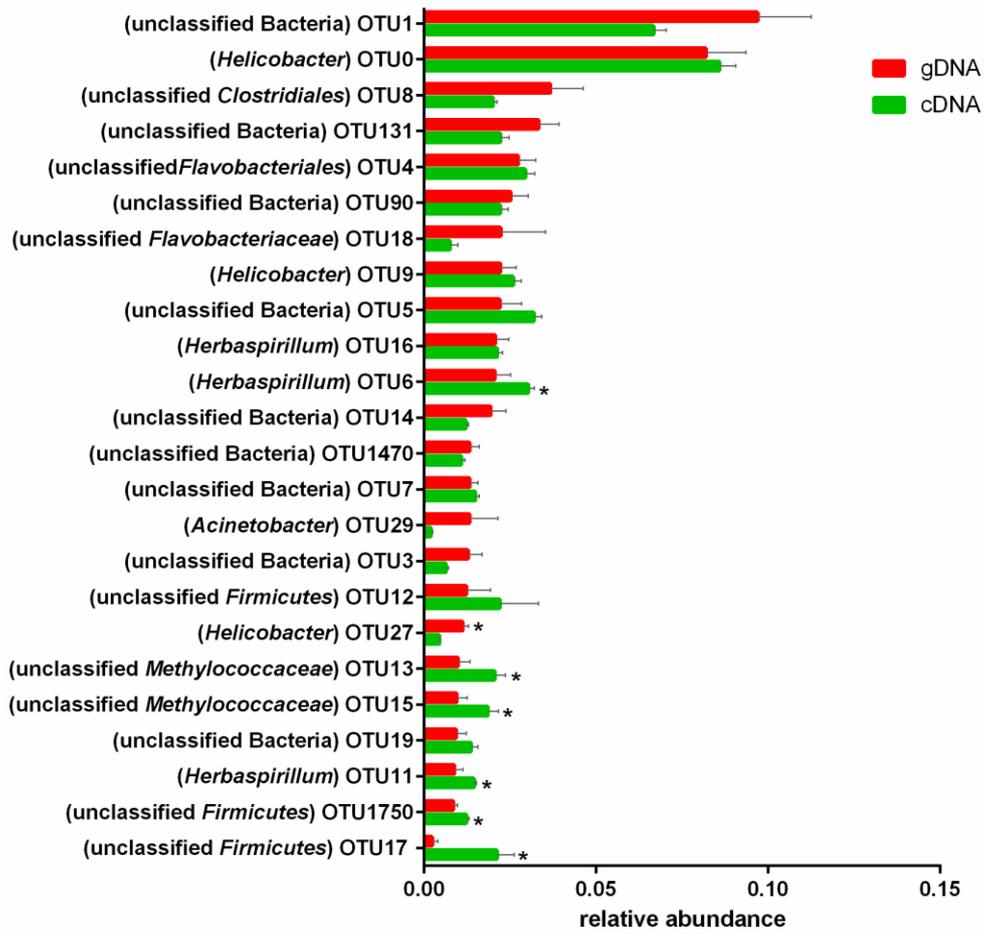
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439   extend to 1.5 times the inter quartile range. * *P* < 0.05.
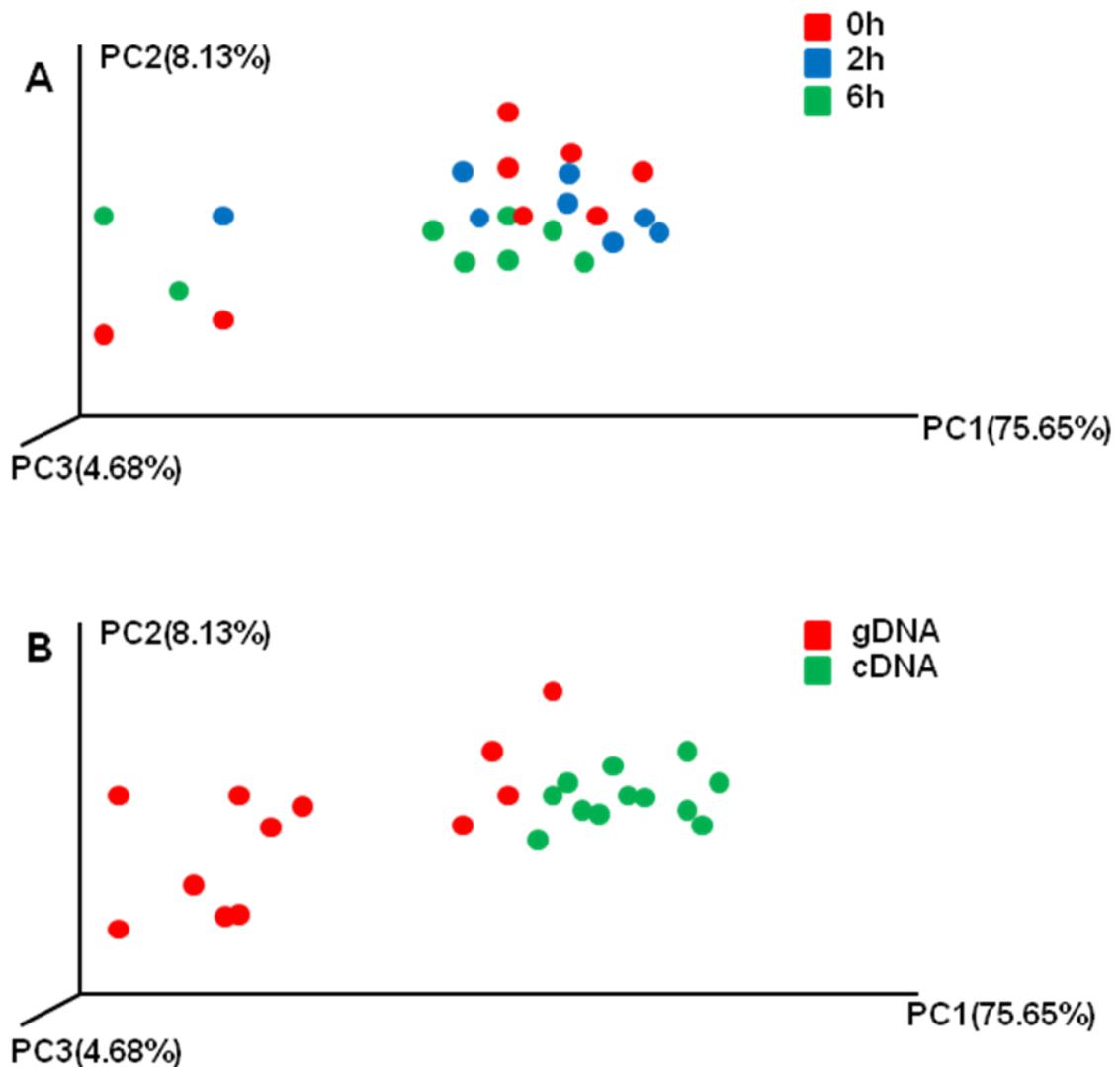
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446 7 genera based on relative abundance are presented, the remaining genera grouped as

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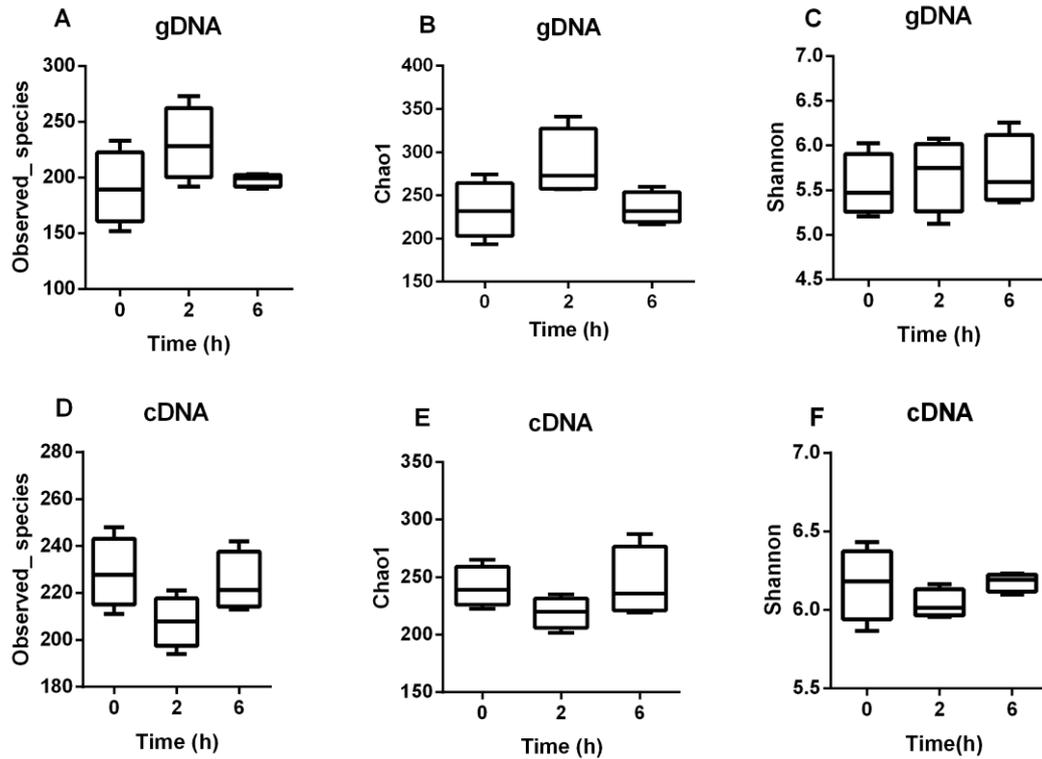**Fig. 4** The relative abundance of top 20 *ureC* gene OTUs. In brackets represent bacteria genus. * *P* < 0.05.
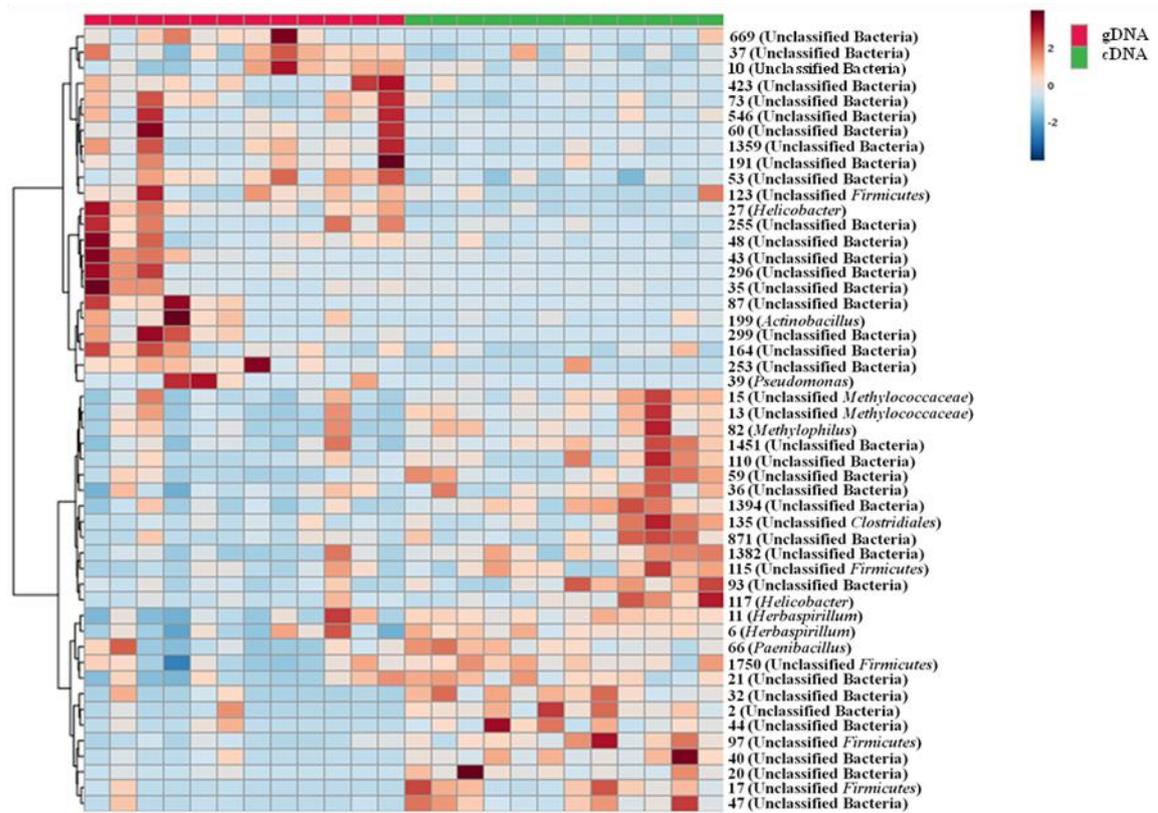
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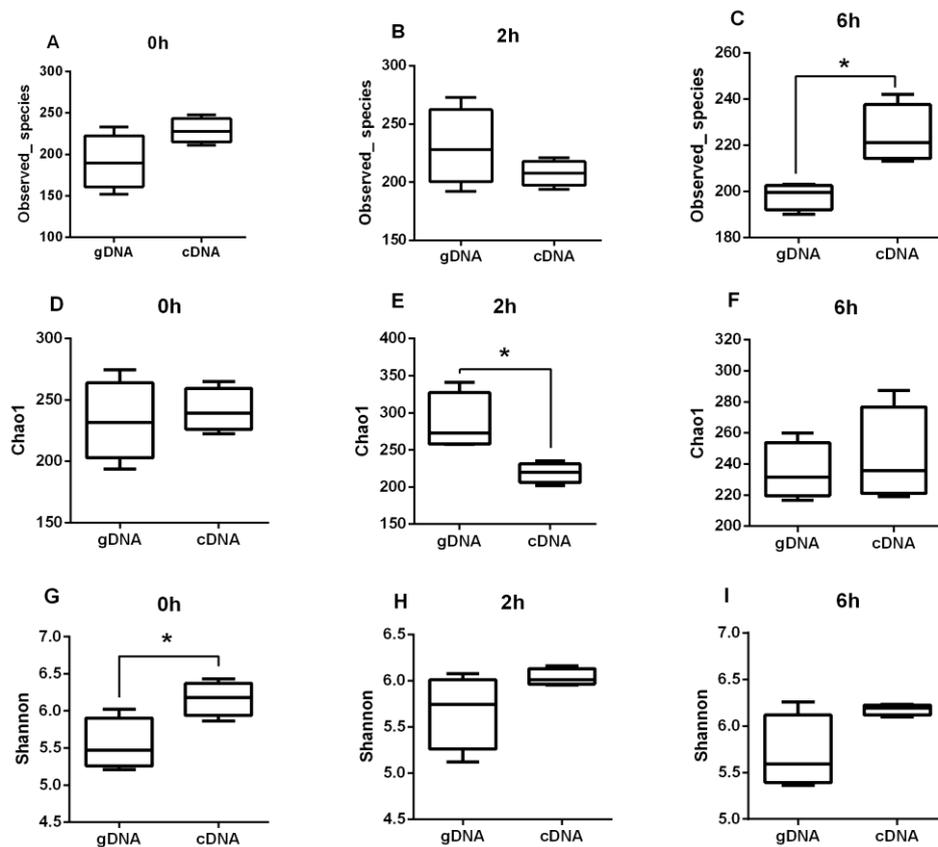
Additional files

Additional file. 1 Alpha diversity of rumen ureolytic bacterias of gDNA and cDNA across different time. (A) Total observed species (B) Chao1 and, (C) Shannon index on gDNA. (A) Total observed species (B) Chao1 and, (C) Shannon index on cDNA. Boxplots indicate the first and third quartiles with the median value indicated as a horizontal line the whickers extend to 1.5 times the inter quartile range.

Additional file. 2 Alpha diversity of rumen ureolytic bacterias across gDNA and cDNA at different time. (A) Total observed species (B) Chao1 and, (C) Shannon index on at 0h. (D) Total observed species (E) Chao1 and, (F) Shannon index on at 2h. (G) Total observed species (H) Chao1 and, (I) Shannon index at 6h. Boxplots indicate the first and third quartiles with the median value indicated as a horizontal line the whickers extend to 1.5 times the inter quartile range. * $P < 0.05$.

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