1 **Title:** Identification of bacteria involved in the decomposition of lignocellulosic biomass treated with
2 cow rumen fluid by metagenomic analysis

3

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23

24 **Abstract**

25 Previously, pretreatment of plant biomass using rumen fluid systems was developed to decompose cell
 26 wall. However, microbes which involved in plant cell wall decomposition in this system have not been
 27 identified, because the conditions of this system are different from the *in situ* rumen environment. We
 28 investigated the bacteria involved in the decomposition of cellulose and hemicellulose in a waste paper
 29 with the rumen pretreatment system using shotgun metagenomic analysis with next generation
 30 sequencing. After pretreatment of waste paper, about a half of the cellulose and hemicellulose content
 31 was decomposed. Genes encoding for cellulase and hemicellulase were mainly found to belonging to
 32 *Ruminococcus*, *Clostridium*, and *Exiguobacterium*. This study shows that *Clostridium* and
 33 *Exiguobacterium*, which have not been identified as predominant genus involved in cellulose and
 34 hemicellulose decomposition, might be categorized as the main fibrolytic bacteria in this system.

35

36 **Key words**

37 *Clostridium*, Cellulase, *Exiguobacterium*, *Ruminococcus*, Hemicellulase

38

39 **Introduction**

40 The development of carbon-neutral biomass conversion processes has attracted interest in recent years,
 41 promising an alternative platform for the production of fuels and chemicals that could reduce the global
 42 dependence on petroleum [7]. Lignocellulosic plant biomass is the most abundant carbon source on the
 43 earth has potential to be a renewable carbon [27]. The plant cell wall is composed of three closely related
 44 polymers: cellulose, hemicellulose, and lignin. They form a physical barrier that inhibits the adsorption of
 45 hydrolytic enzymes and protects them. For effective carbon utilization, plant biomass has been pretreated
 46 with wet oxidation and chemical methods so the plant cell wall can be hydrolyzed to monosaccharide [14,
 47 38]. However, wet oxidization requires a substantial amount of energy and chemical methods developed
 48 so far are inefficient for lignocellulose decomposition [9, 21].

49 Ruminant animals efficiently utilize plant materials as an energy source because they can decompose
 50 plant cell walls in the rumen. Microorganisms in the rumen play an important role in hydrolysis and
 51 fermentation of lignocellulose [19, 20]. Several studies have indicated that rumen microbes can degrade a
 52 larger amount of cellulose in less time than anaerobic digester [25, 31]. Baba et al. [2] developed an
 53 effective lignocellulosic biomass decomposition method that uses rumen fluid. It can decompose more
 54 than half of the cellulose, hemicellulose, and lignin content by pretreating with rumen fluid over 24 h.

55 Baba et al. [1] further discovered that the bacterial community changes during the pretreatment period by
56 16S rRNA amplicon sequencing analysis. While, this analysis has been clarified for the microbial
57 composition, but it did not explicitly identify the microorganisms involved in the decomposition process.
58 Shotgun metagenomic analysis randomly sequences the DNAs present in environmental samples using a
59 next-generation sequencer, which provides an effective method to determine microbial composition and
60 general function from uncultivated microorganisms [33]. Several studies have conducted metagenomic
61 analysis in cow rumen and revealed the several microbes that are involved in the decomposition of the
62 plant cell wall decomposition [3, 5]. However, the pretreatment process is an unbuffered closed system
63 that treats a single substrate. Further complicating the system, different microbes may be involved in the
64 *in vitro* decomposition process compared with *in situ* cow rumen environment. Thus, we conducted a
65 shotgun metagenomic study on the lignocellulosic biomass pretreatment with rumen fluid system to
66 reveal the cellulose and hemicellulose decomposing microbes. This study shows that unique this system
67 may utilize unique microbes compared to the *in situ* rumen environment to decompose the plant cell wall
68 in waste paper.

69

70 **Material and Method**

71 **Pretreatment of wastepaper using rumen fluid**

72 The treatment condition followed the method of Baba et al. [2]. Rumen contents were collected orally
 73 from grass-fed cattle and filtered with a mesh strainer (1 mm × 1 mm) to remove coarse solids. 0.3g of
 74 the waste paper pieces (35 mm long × 25 mm wide) were soaked in 300 ml of rumen fluid containing 0.3
 75 g of L-cysteine as a reducing agent. They were incubated at 37 °C on a rotary shaker at 140 rpm for 120 h.
 76 Fluid samples of the reaction were collected before and after 6, 24, 48, 60, 96 and 120 h of pretreatment
 77 and conducted to chemical analysis. The experiments were conducted in duplicate.

78

79 **Chemical composition of the waste paper and pretreatment systems**

80 The cellulose, hemicellulose, and lignin contents in the waste paper were determined according to the
 81 detergent fiber method [34]. The chemical composition of the waste paper was 71.3% cellulose, 18.5%
 82 hemicellulose, and 8.9% lignin. Volatile fatty acid (VFA) concentrations were determined using
 83 LC-10Avp Typ HPLC with an ultraviolet detector (Shimadzu, Kyoto, Japan), equipped with an
 84 ion-exchanger column RSpak KC-811 (Shodex, Tokyo, Japan). Analysis were performed at 60 °C using 3
 85 mM HClO₄ for elution. The dissolved chemical oxygen demand (dCOD) was measured by the dichromate
 86 reactor digestion method [15].

87

88 **DNA extraction from rumen fluid**

89

90 The fluid samples collected from before and after 60 h pretreatment were used for DNA extraction

91 because microbes actively decompose substrates in this time point (see results). DNA was extracted from

92 microcosms according to the method of Morita et al. [23]. In brief, bacterial DNA was isolated by the

93 enzymatic lysis method. Cells were lysed in the presence of lysozyme, achromopeptidase, and Proteinase

94 K. Cleared lysate was treated with phenol/chloroform/isomyl alcohol and RNase. DNA was precipitated

95 with ethanol and suspended dissolved in Tris-EDTA (TE) buffer. DNA was quantified using PicoGreen™

96 dsDNA Assay Kit (Thermo Scientific Massachusetts, USA). The integrity of DNA was verified by a

97 Nanodrop (Thermo Scientific, Massachusetts, USA) and gel visualization (0.8 % agarose in Tris-acetate

98 EDTA buffer).

99

100 **Shotgun metagenome sequencing**

101 DNA Libraries were subjected to paired-end sequencing on an Illumina MiSeq sequencer (Illumina,

102 SanDiego, CA, USA) at the Biotechnology Center, Akita Prefectural University (Akita, Japan) with

103 Miseq Reagent Kit v2 500 cycles (~ 250 bp) using Truseq library kit (Illumina) according to the
104 manufacturer's instructions. The information obtained by sequencing is shown in Table S1. The
105 sequencing data were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under
106 accession number is DRA007782.

107

108 **Data analysis**

109 Paired-end sequences were joined together with > 30 bp overlapping sequences containing < 10%
110 mismatches and then converted from fastq to the fasta format with a Q-score cutoff of > 30 using
111 PRINSEQ [29]. Ribosomal RNA sequences (rRNAseq) in the metagenomics data were identified using a
112 BLAST search against the M5RNA database (E value of $< 10^{-5}$, alignment length of >50 bp) on the
113 MG-RAST server ver. 3.6 [22]. Microbial diversity and richness were shown as Shannon index, operation
114 taxonomic units (OTU) numbers, and Chao1 as index determined by MG-RAST. We collected highly
115 abundant gene sequences of key enzymes involved in cell wall decomposition according to the KEGG
116 compound database [16]. The identified sequences were compared to the NCBI nr database in December
117 2015 by BLASTX for more precise assignment (E value of $< 10^{-5}$, alignment length of >30 aa).

118

119 **Results**

120 **Pretreatment of lignocellulosic biomass using rumen fluid**

121 Approximately, half of the cellulose, hemicellulose, and lignin in waste paper reactions were
 122 decomposed after 60 h of pretreatment using rumen fluid (Table 1). The decomposition increased dCOD
 123 and two of the three majored VFAs, which correlated with a decreasing pH value (Figure 1) similar to
 124 Baba et al. [1, 2] using waste paper and rapeseed as substrates. VFA concentrations and dCOD were
 125 continuously increased during pretreatment until 120 h. These results indicated that microbes were
 126 actively decomposing the waste paper at the 60 h time point.

127

128 **Alteration of microbial community, diversity, and richness in the pretreatment system**

129 The microbial communities residing in the rumen fluid changed during the incubation period (Figure 2).
 130 At the phylum level, Firmicutes were increased from 53.4 and 65.5 % before information to 66.5 and
 131 75.0 % after incubation in test sample one and two, respectively. At the class level *Clostridia*, a class of
 132 Firmicutes represented 36.4 and 50.3 % before incubation to 50.3 and 63.6 % after incubation. The
 133 relative abundance of other classes of microbes, including Bacilli, Bacteroida and Gammaproteobacteria,
 134 were decreased. At the genus level, *Ruminococcus*, *Eubacterium*, and *Butyrivibrio*, all classes of

135 *Clostridia*, were increased (Table 2). Contrastingly, *Bacillus* and *Prevotella* were decreased after
136 pretreatment. The increasing of *Clostridia* and decreasing other microbes caused the microbial diversity
137 and richness decreasing (Table 3). Microbial diversity (Shannon index) was decreased to about 0.9 times
138 while, microbial richness (OTU number and Chao1) more than 0.5 times decreased after pretreatment.

139

140 **Prediction of gene functions and microbes involved in plant cell wall degradation**

141 The functional binning of shotgun metagenomics (based on KEGG orthology) indicated that
142 predominant functions detected in the rumen microbiota were metabolism (triphosphate reductase,
143 phosphatase, etc.) at 56.8 % and genetic information processing (RNA polymerase, RNA synthetase etc.)
144 at 25.5 %. Other functional assignments were linked to environmental information processes (11.8 %)
145 and cellular processes (4.5 %).

146 We analyzed three genes encoding the proteins involved in cellulase and hemicellulase activities as
147 beta-glucosidase (EC 3.2.1.21 and EC 3.2.1.74), beta-glucanase (EC 3.2.1.4), and endoxylanase (EC
148 3.2.1.8). Cellulose degradation (beta-glucosidase and beta-glucanase) collectively represented about
149 0.123 % of total sequence reads. The beta-glucosidase coding genes were mostly from bacterial genera
150 *Butyrivibrio*, *Ruminococcus*, *Clostridium*, and *Exiguobacterium* (Figure 3). Interestingly, the relative

151 abundance of *Exiguobacterium* was increased to about 100-fold and 40-fold in the sample after
152 pretreatment, while that from *Bacillus*, *Butyrivibrio*, and *Prevotella* were decreased. Beta-glucanase was
153 mainly affiliated with *Butyrivibrio*, *Clostridium*, and *Ruminococcus*. *Ruminococcus* genes were not
154 detected in the rumen fluid prior to treatment. These genes increased while those from *Butyrivibrio*
155 decreased after pretreatment. Endoxylanase was mainly assigned to *Clostridium* and *Ruminococcus* and
156 both increased after pretreatment (Figure 4).

157

158 Discussion

159 In this study, we conducted metagenomic analysis to reveal the microbes involved in lignocellulose
160 decomposition in the pretreatment system by cow rumen fluid. Culture-independent methods using a
161 next-generation sequencer have been applied to study bacteria involved in plant cell wall degradation *in*
162 *situ* cow rumen [3, 5, 11, 35]. In this study, we showed that *Ruminococcus* and *Clostridium*, they are
163 belonging to *Clostridia*, were the primary cellulolytic and hemicellulolytic bacteria. Baba et al. [1]
164 showed that after 24 h rapeseed (*Brassica napus* L.) pretreatment *Clostridia*, especially *Ruminococcus*,
165 increased while *Bacteroidetes* decreased. Thus, *Clostridia* flourishing in the pretreatment system appear
166 to be independent of substrate types. Microbial diversity and richness were decreased along with

167 increasing of Clostridial abundance. *Clostridia* are gram-positive, anaerobic bacteria found in rumen that
168 are capable of degrading plant cell walls [24, 37]. They are also known to be fibrolytic and several
169 species have been isolated from rumen. *R. albus* and *R. flavefaciens*, are considered the major cellulolytic
170 bacteria in the rumen [5, 10, 28, 36]. They also display degradation properties against of filter paper. In
171 addition to total organism abundance, the cellulase and hemicellulase genes from *Clostridium* also
172 increased after pretreatment. *C. thermocellum* and *C. aldrichii* can associated to the plant cell wall and
173 have a cellulosome, which is a multienzyme complex specialized for cellulose degradation [18]. After the
174 pretreatment, the beta-glucosidase genes affiliated with *Exiguobacterium* also increased.
175 *Exiguobacterium* has been detected with molecular biological methods but has not been isolated from
176 rumen [5, 13]. They have been isolated from permafrost cores, soils, and several water environments, and
177 they grow between -2.5 to 40°C [26]. *Exiguobacterium* possess alpha-glucosidase, beta-glucosidase, and
178 beta-galactosidase genes that enable them to decompose cellobiose and carboxymethyl cellulose [12, 26].
179 This is the first result showing the detection of the cellulase genes and their involvement in cellulose
180 degradation from a cow rumen environment. *Clostridium* and *Exiguobacterium* are not dominant
181 organisms (representing 5.0 and 0.01 % of *in situ* rumen flora, respectively) and not mainly involved in
182 cellulose and hemicellulose degradation *in situ* rumen environment [3, 5]. However, they might mainly

183 contribute to lignocellulose degradation in the pretreatment system despite they were not dominant. Thus,
184 the detection of them as main lignocellulosic microbes *in vivo* rumen was unique finding compared with
185 previous studies.

186 Plant cell wall degradation process *in situ* rumen is primarily attributed to *Ruminococcus*, *Prevotella*,
187 *Butyrivibrio*, and *Fibrobacter* [3, 5, 19]. However, the genes encoding to cellulase and hemicellulase
188 assigned to *Prevotella* and *Butyrivibrio*, were decreased after incubation. *Prevotella* belonging to
189 *Bacteroidetes* are well known to be involved in decomposition of lignocellulosic biomass in a rumen [18,
190 30]. They can digest starch, hemicellulose, and pectin [4, 6]. *P. ruminicola* is a *Prevotella* strain found in
191 rumen with endoxylanase and beta-glucosidase that can decompose filter paper [8, 20, 24]. *B.*

192 *fibrisolvens* is also recognized as the main hemicellulolytic bacteria in the rumen [20]. Moreover, *P.*

193 *ruminicola* and *B. fibrisolvens* can accelerate the cellulose digestion rate combined with fibrolytic

194 bacteria such as *Ruminococcus* or *Fibrobacter* [17]. It has been reported that the quantities of *P.*

195 *ruminicola* are increased significantly when the animal's diet is switched from a hay to high grain, but

196 that the abundance of *R. flavefaciens*, and *B. fibrisolvens* organisms are reduced [32]. Cellulose is the

197 main components in waste paper, thus, non-cellulolytic *Prevotella* might be expelled due to the

198 dominance of cellulolytic *Ruminococcus*, *Clostridia*, and *Exiguobacterium*. The 16S rRNA based relative

199 abundance of *Butyrivibrio* was increased after pretreatment, but functional genes-based cellulolytic
200 *Butyrivibrio* was decreased. We were suggested that *Butyrivibrio* is not involved in cellulose or
201 hemicellulose degradation, because *B. hungatei* or unknown *Butyrivibrio* spp. were increased.
202 In conclusion, we showed *Ruminococcus* are primary members involved in plant cell wall decomposition,
203 in agreement with previous studies. On the other hand, *Clostridium* and *Exiguobacterium*, which were
204 primary fibrolytic bacteria in the previous studies, might play a more significant role in the decomposition
205 of cellulose and hemicellulose in waste paper. Moreover, the main fibrolytic bacteria *in situ* rumen,
206 *Prevotella* and *Butyrivibrio*, were also decreased. These results suggested the rumen pretreatment system
207 environment was different to from the *in situ* rumen environment, and unique microbes were contributed
208 degradation of lignocellulosic biomass. Future research will develop an effective decomposition system
209 making use of *Ruminococcus*, *Clostridium*, and *Exiguobacterium*.

210

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219

220 **Compliance with ethical standards**

221 **Conflict of interest**

222 The authors declare that they have no conflict of interest.

223

224 **Ethical approval**

225 All experimental procedures conformed to "Regulations for Animal Experiments And Related Activities

226 at Tohoku University", and were reviewed by the Institutional Laboratory Animal Care and Use

227 Committee of Tohoku University, and finally approved by the President of University.

228

229 **References**

- 230 1. Baba Y, Matsuki Y, Mori Y, Suyama Y, Tada C, Fukuda Y, Saito M, Nakai Y (2017) Pretreatment
231 of lignocellulosic biomass by cattle rumen fluid for methane production: Bacterial flora and enzyme
232 activity analysis. J Biosci Bioeng. 123: 489-496. doi: 10.1016/j.jbiosc.2016.11.008
- 233 2. Baba Y, Tada C, Fukuda Y, Nakai Y (2013) Improvement of methane production from waste paper
234 by pretreatment with rumen fluid. Bioresour Technol. 128, 94-99. doi:
235 10.1016/j.biortech.2012.09.077
- 236 3. Comtet-Marre S, Parlsot N, Lepercq P, Chaucheyras-Durand F, Mosoni P, Peyretallade E, Bayat AR,
237 Shingfield KJ, Peyret P, Frano E (2017) Metatranscriptomic reveals the active bacterial and
238 eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. Frontiers in Microbiol.
239 Jan 31;8:67. doi: 10.3389/fmicb.2017.00067.
- 240 4. Cotta MA. (1992) Interaction of ruminal bacteria in the production and utilization of
241 maltooligosaccharides from starch. Appl Environ Microbiol. 59:48-54.
- 242 5. Dai X, Tian Y, Li J, Su X, Wang X, Zhao S, Liu L, Luo Y, Liu D, Zheng H, Wang J, Dong Z, Hu S,
243 Huang L. (2015) Metatranscriptomic analysis of plant cell wall polysaccharide degradation by
244 microorganisms in the cow rumen. Appl Environ Microb, 81:1375-1385. doi:
245 10.1128/AEM.03682-14.

- 246 6. Dehorty BA (1991) Effects of microbial synergism on fiber digestion in the rumen. *Proc Nutr Soc.*
247 50:149-159.
- 248 7. FitzPatrick M, Champagne P, Cunningham MF, Whitney RA. (2010) A biorefinery processing
249 perspective: Treatment of lignocellulosic materials for the production of value-added products.
250 *Bioresour Technol.* 101:8915-8922. doi: 10.1016/j.biortech.2010.06.125.
- 251 8. Fox DG, Barry MC, Pitt RE, Roseler DK, Stone WC. (1995) Application of cornell net carbohydrate
252 and protein model for cattle consuming forages. *J Anim Sci.* 73:267-277.
- 253 9. Fox M, Noike T (2004) Wet oxidation pretreatment for the increase in anaerobic biodegradability of
254 newspaper west. *Bioresour Technol.* 91:273-281. doi: 10.1016/j.biortech.2003.06.001.
- 255 10. Forsberg CW, Cheng KJ, White BA. (1997) Polysaccharide degradation in the rumen and large
256 intestine. In: Mackie RI, White BA, Isacsen RE (ed) *Gastrointestinal Microbiology*. Vol.1.
257 Chapman and Hall, New York. pp 319-379.
- 258 11. Güllert S, Fischer MA, Turaev D, Noebauer B, Ilmeberger N, Wemheuer B, Alawi M, Rattei T,
259 Daniel R, Schmitz RA, Grundhoff A, Streit WR. (2016) Deep metagenome and metatranscriptome
260 analysis of microbial communities affiliated with an industrial biogas fermenter, a cow rumen, and

261 elephant feces reveal major differences in carbohydrate hydrolysis strategies. *Biotechnol Biofuels*

262 9:121. doi: 10.1186/s13068-016-0534-x.

263 12. Hagaggi NSA. (2018) Biodegradation of cellulosic raw materials by extracellular carboxymethyl

264 cellulase produced by *Exiguobacterium aurantiacum*. *Egypt J Biol.* 14:11-18. doi:

265 10.5455/egyjebb.20170807070652.

266 13. Hattori K, Matsui H. (2008) Diversity of fumarate reducing bacteria in the bovine rumen revealed

267 by culture dependent and independent approach. *Anaerobe.* 14:87-93. doi:

268 10.1016/j.anaerobe.2007.12.002.

269 14. Hendriks ATWM, Zeeman G. (2009) Pretreatments to enhance the digestibility of lignocellulosic

270 biomass. *Bioresour Technol.* 100:10-18. doi: 10.1016/j.biortech.2008.05.027.

271 15. Jirka AM, Carter MJ. (1975) Micro semi-automated analysis of surface and wastewaters for

272 chemical oxygen demand. *Anal Chem.* 47:1397.

273 16. Kaneshima M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M,

274 HirakawaM. (2006) From genomics to chemical genomics: new developments in KEGG. *Nucleic*

275 *Acids Res.* 31:371-373. doi: 10.1093/nar/gkj102.

- 276 17. Kim M, Morrison M, Yu Z. (2011) Phylogenetic diversity of bacterial communities in bovine rumen
277 as affected by diets and microenvironment. *Folia Microbiol.* 56:453-458. doi:
278 10.1007/s12223-011-0066-5.
- 279 18. Koike S, Yoshitani S, Kobayashi Y, Tanaka K. (2003) Phylogenetic analysis of fiber-associated
280 rumen bacterial community and PCR detection of uncultured bacteria. *FEMS Microbiol Lett.*
281 229:23-30. doi: 10.1016/S0378-1097(03)00760-2.
- 282 19. Koike S, Kobayashi Y. (2009) Fibrolytic rumen bacteria: Their ecology and functions. *Asian-Aust J*
283 *Anim Sci.* 22:131-138. doi: 10.5713/ajas.2009.r.01.
- 284 20. Kurause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, McSweeney CS. (2003)
285 Opportunities to improve fiber degradation bacteria in the rumen: microbiology, ecology, and
286 genomics. *FEMS Microbiol Rev.* 27:663-693.
- 287 21. Lin Y, Wang D, Wu S, Wang C. (2009) Alkali pretreatment enhances biogas production in the
288 anaerobic digestion of pulp and paper sludge. *J Hazard Mater.* 170:366-373. doi:
289 10.1016/S0168-6445(03)00072-X.
- 290 22. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Roderiguez A,
291 Stevens R, Wilke A, Wilkening J, Edwards RA. (2008) The metagenomics RAST server – a public

292 resource for the automatic phylogenetic and functional analysis of metagenomics. BMC
293 Bioinformatics. 19;9:386. doi: 10.1186/1471-2105-9-386

294 23. Morita H, Kuwahara T, Ohshima K, Sasamoto H, Itoh K, Hattori M, Hayashi T, Takami H. (2007)
295 An improved DNA isolation method for metagenomic analysis of the microbial flora of human
296 intestine. Microbes Environ. 22:214-222. doi: 10.1264/jsme2.22.214

297 24. Nyonyo T, Shinkai T, Mitsumori M. (2014) Improved culturability of cellulolytic rumen bacteria
298 and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the
299 bovine rumen. FEMS Microbiol Ecol. 88:528-537. doi: 10.1111/1574-6941.12318.

300 25. O'Sullivan CA, Burrell PC, Clarke WP, Blackall LL. (2006) Comparison of cellulose solubilisation
301 rates in rumen and landfill leachate inoculated reactors. Bioresour Technol. 97:2356-2363.
302 doi:10.1016/j.biortech.2005.10.021.

303 26. Rodrigues DF, Goris J, Vishnivetskaya T, Gilichinsky D, Thomashow Mf, Tiedje JM. (2006)
304 Characterization of *Exiguobacterium* isolates from the Siberian permafrost. Description of
305 *Exiguobacterium sibiricum* sp. nov. Extremophiles. 10:285-294. doi: 10.1007/s00792-005-0497-5.

306 27. Rubin EM. (2008) Genomics of cellulosic biofuels. Nature. 454:841-845. doi:
307 10.1038/nature07190.

- 308 28. Russel JB, Muck RE, Weimer PJ. (2008) Quantitative analysis of cellulose degradation and growth
309 of cellulolytic bacteria in the rumen. FEMS Microbiol Ecol. 67:183-197. doi:
310 10.1111/j.1574-6941.2008.00633.x.
- 311 29. Schmieder R, Edwards R. (2011) Quality control and preprocessing of metagenomic datasets.
312 Bioinformatics. 27:863-864. doi: 10.1093/bioinformatics/btr026.
- 313 30. Shinkai T, Kobayashi Y. (2007) Localization of ruminal cellulolytic bacteria on plant fibrous
314 materials as determined by fluorescence in situ hybridization and real-time PCR. Appl Environ
315 Microbiol. 73:1646-1652. doi:10.1128/AEM.01896-06.
- 316 31. Song H, Clarke WP, Blackall LL. (2005) Concurrent microscopic observations and activity
317 measurements of cellulose hydrolyzing and methanogenic populations during the batch anaerobic
318 digestion of crystalline cellulose. Biotechnol Bioeng. 91:369-378. doi:10.1002/bit.20517.
- 319 32. Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. (2001) Diet-dependent shifts
320 in the bacterial population of the rumen revealed with real-time PCR. Appl Environ Microbiol.
321 67:2766-2774. doi: 10.1128/AEM.67.6.2766-2774.2001

- 322 33. Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram J, Richardson PM, Solovyev VV, Rubin EM,
323 Rokhsar DS, Banfield JF. (2004) Community structure and metabolism through reconstruction of
324 microbial genomes from the environment. 428:37-43. doi: 10.1038/nature02340
- 325 34. Van Soest PJ, Wine RH. (1968) Determination of lignin and cellulose in acid detergent fiber with
326 permanganate. J. Assoc. Off. Agric. Chem. 51, 780-785.
- 327 35. Wang L, Hatem A, Catalyurek UV, Morrison M, Yu Z. (2013) Metagenomic insights in to the
328 carbohydrate-active enzymes carried by the microorganisms adhering to solid digesta in the rumen
329 of cow. Plos One. 8:e78507. doi: 10.1371/journal.pone.0078507.
- 330 36. Weimer PJ, Conner AH, Lorenz LF. (2003) Solid residues from *Ruminococcus* cellulose
331 fermentations as components of wood adhesive formulations. Appl Microbiol Biotechnol. 63:29-34.
332 doi: 10.1007/s00253-003-1334-3
- 333 37. Weimer PJ, Koegel RG, Lorenz LF, Frihart CR, Kenealy WR. (2005) Wood adhesives prepared
334 from lucerne fiber fermentation residues of *Ruminococcus albus* and *Clostridium thermocellum*.
335 Appl Microbiol Biotechnol 66:635-640. doi: 10.1007/s00253-004-1767-3.

- 336 38. Zhu JY, Pan XJ. (2010) Woody biomass pretreatment for cellulosic ethanol production. Technology
337 and energy consumption evaluation. Bioresour Technol 101:4992-5002. doi:
338 10.1016/j.biortech.2009.11.007.

339 **Table 1. Chemical components (%) of waste paper before and after pretreatment**

340

	0 h	60 h	Decomposition rate
Cellulose	71.3	30.3	57.4
Hemicellulose	18.5	9.69	47.6
Lignin	8.9	5.09	42.7

341

342

343 Table 2. The relative abundance of top 20 genus and *Exiguobacterium* before and after

344 pretreatment

345

	0 h		60 h	
	1	2	1	2
<i>Ruminococcus</i>	5.50%	6.70%	26.40%	16.60%
<i>Clostridium</i>	13.20%	14.90%	12.80%	11.70%
<i>Eubacterium</i>	3.70%	4.80%	6.20%	6.20%
<i>Bacteroides</i>	3.50%	3.70%	1.40%	2.50%
<i>Butyrivibrio</i>	0.80%	2.40%	3.30%	3.70%
<i>Methanobrevibacter</i>	4.10%	2.30%	1.90%	1.80%
<i>Streptococcus</i>	3.60%	2.40%	1.20%	2.20%
<i>Bacillus</i>	3.80%	2.30%	1.50%	1.70%
<i>Prevotella</i>	2.70%	2.80%	1.10%	2.30%
<i>Blautia</i>	1.70%	3.90%	1.30%	1.50%
<i>Lactobacillus</i>	1.50%	1.40%	0.60%	0.90%
<i>Roseburia</i>	0.58%	0.93%	1.36%	1.42%
Unclassified	0.52%	1.31%	1.07%	0.99%
<i>Clostridiales</i>				
<i>Faecalibacterium</i>	0.84%	1.73%	0.54%	0.61%
<i>Acidaminococcus</i>	0.48%	0.96%	1.21%	0.94%
Unclassified	1.15%	2.08%	0.13%	0.12%
<i>Siphoviridae</i>				
<i>Coprococcus</i>	0.49%	0.85%	0.88%	1.19%
<i>Slackia</i>	1.00%	1.24%	0.46%	0.56%
Unclassified	0.70%	1.26%	0.56%	0.66%
<i>Lachnospiraceae</i>				
Unclassified	0.61%	0.80%	0.70%	0.71%
<i>Peptostreptococcaceae</i>				
<i>Exiguobacterium</i>	0.05%	0.06%	0.09%	0.11%

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Table 3. Microbial diversity and richness before and after pretreatment

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	0 h		60 h	
	1	2	1	2
Shannon index	6.16	6.21	5.7	5.3
OTU number	2102	2223	2052	1011
Chao1	482	414	266	178

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351 **Figure captions**

352 Figure 1. Chemical properties of the rumen fluid system during the incubation period.

353 (a) pH; (b) Volatile fatty acids concentration; (c) Dissolved chemical oxygen demand (dCOD)

354 concentration

355

356 Figure 2. Relative abundance of microbial community (phylum and class level) during pretreatment.

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358 Figure 3. Relative abundance and taxonomic affiliation of cellulase (beta-glucosidase and beta-glucanase)

359 during the incubation period.

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361 Figure 4. Relative abundance and taxonomic affiliation of hemicellulose (endoxy lanase) during the

362 incubation period.

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Figure 2

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Figure 3

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Figure 4

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Table S1. Results of shotgun metagenome sequencing analysis

Pretreatment period	0 h		60 h	
Sample number	1	2	1	2
Total reads (bp)	877,861,769	1,383,852,122	566,411,425	67,903,534
Contig count	144,532	237,377	110,992	8,919
Total length (bp)	107,766,578	183,903,617	72,839,544	5,332,988
Mean sequence length	746±1115	775±1250	656±558	598±1041
Features	54,170	95,259	97,315	8,438
Predicted protein features	176,063	287,751	129,323	10,605
Predicted rRNA features	13,685	20,986	11,151	796

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