Canadian Journal of Microbiology

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2021-0338.R1
Manuscript Type:	Article
Date Submitted by the Author:	31-Dec-2021
Complete List of Authors:	Yang, Jie; University of Chinese Academy of Sciences; Ghent University Zhao, Jie; University of Chinese Academy of Sciences Wang, Bobo; University of Chinese Academy of Sciences Yu, Zhisheng; University of Chinese Academy of Sciences
Keyword:	Aerobic cultivation, cellulolytic microbes, gastrointestinal tract, sheep, enzyme activity
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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Unraveling aerobic cultivable cellulolytic microorganisms within the gastrointestinal tract of sheep (*Ovis aries*) and their evaluation for cellulose biodegradation

Running headline: Aerobic cellulolytic microbes of sheep GT

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Abstract

Anaerobic cellulolytic microbes in gastrointestinal tract (GT) of ruminants have been well-documented, however, knowledge of aerobic microbes with cellulolytic activities in ruminant GT is comparably limited. Here, we unraveled aerobic cultivable cellulolytic microbes in GT of Ujimqin sheep (*Ovis aries*) and evaluated the cellulolytic potential of promising isolates. Twenty-two strains were found to possess cellulose degrading potential by Congo-red staining and phylogenetic analysis of the 16S rDNA/ITS sequence revealed that all strains belonged to nine genera, i.e., *Bacillus*, *Streptomyces*, *Pseudomonas*, *Lactobacillus*, *Brachybacterium*, *Sanguibacter*, *Rhizobium*, *Fusarium*, and *Aspergillus*. Strains with high cellulolytic activities were selected to further evaluate the various enzyme activities on lignocellulosic alfalfa hay (*Medicago sativa*). Among them, isolate *Bacillus subtilis* RE2510 showed the highest potential of cellulose degradation considering the high endoglucanase (0.1478 ± 0.0014 IU ml⁻¹), exoglucanase (0.1735 ± 0.0012 IU ml⁻¹) and β -glucosidase (0.3817 ± 0.0031 IU ml⁻¹) after 10-day incubation with alfalfa hay. A significant destruction effect of the cellulose structure and the attachment of *B. subtilis* RE2510 to the hay were also revealed by using scanning electron microscope. This study expands our knowledge of aerobic cellulolytic isolates from GT of sheep and also highlights their potential application as microbial additive in the aerobic process of cellulose bioconversion.

Keywords: Aerobic cultivation, cellulolytic microbes, gastrointestinal tract, sheep, enzyme activity

Introduction

Cellulosic biomass has been acknowledged as one of the promising and renewable energy sources to alleviate the increasing demand for alternative fuel sources. However, the highly polymerized structure, i.e., the linear polymer chains linked by β -1,4 glucosyl bonds as well as the surrounding hemicellulose and lignin (Gardner and Blackwell 1974; Navarro et al. 2019), increased the difficulty in degradation and bioconversion of this cellulose complex. Owing to the multiple cellulolytic enzymes produced by cellulolytic bacteria and fungi, significant emphasis has been placed on the exploration of high efficiency and accessibility cellulolytic microbes in natural environments. Over recent decades, cellulolytic microorganisms have been detected in a wide range of niches, whereas the gastrointestinal tract (GT) of ruminant herbivores is especially attractive for detecting the promising cellulose-degrading bacteria among varied environments (Nyonyo et al. 2014; Sari et al. 2017).

Ruminants and most of herbivores lack the ability to hydrolyze lignocellulose by themselves and instead they can effectively utilize the feed of cellulosic substrates with the aid of cellulolytic and other co-existed microorganisms residing in their GT (Mackie 2002). Accumulating studies have reported the isolates of cellulolytic microbes in GT of ruminants over the previous decades and microbial screening has been extended to a variety of ruminant species, including cattle (Halliwell and Bryant 1963), sheep (Liu et al. 2019), camel (Samsudin et al. 2012), yak (Cai and Dong 2010), and deer (Park et al. 2006). Although diverse cellulolytic microbes with a wide range of cellulolytic potential have been detected in the GT of these ruminants, most of these studies focused on anaerobic cultivable microbes, which leads to a lack of knowledge of aerobic cultivable microorganisms with cellulolytic activities in GT of ruminants. It has been increasingly acknowledged that the ruminant GT is not a strict anaerobic environment as some amounts of oxygen enter the GT along with the feed or diffuse from the

bloodstream through the epithelial cells (Hobson and Stewart 1997; Santra and Karim 2003), which suggested a mechanism to exclusion of obligate anaerobes and extremely oxygen-sensitive microbes within ruminant GT. One recent metatranscriptomic work revealed a substantial proportion of active aerobic fungi in cattle rumen (Zhang et al. 2020), and most of these aerobic fungi were previously reported to be associated with lignocellulosic degradation, which indicated the possibility to screen aerobic cellulolytic microbes within the ruminant GT. However, whether these aerobic microbes could be obtained based on culture-dependent methods and whether these aerobes species can be confirmed to have the ability to degrade the lignocellulosic components remains unclear.

Previous studies have shown that gut (facultative) aerobes can function as oxygen scavengers and digesters of sloughed epithelial cells (Madigan et al. 1997), which play an essential role in establishing a stable GT environment for ruminants. Further exploring the cellulose-degrading potential of these aerobic cultivable microbes in GT can expand the knowledge of the role of gut aerobes and also beneficial for future practical application in lignocellulosic biomass treatment and sustainable agricultural production. For example, facultative bacterium *Bacillus licheniformis*, isolated from the goat rumen, is considered as a good candidate for bioethanol production due to their high production of multiple cellulose- and hemicellulolytic enzymes with cost-effective agricultural residues (Seo et al. 2013; Seo et al. 2014). Li et al. (2018) isolated two *Enterococcus* strains from yak rumen and demonstrated that these strains could be efficient microbial additives during ensiling as they significantly increase the glucose yield and cellulose convertibility of *Pennisetum sinense* silage. Moreover, the aerobic cellulolytic microbes isolated from ruminant GT can also be used as promising biological inoculant in a mixed-culture fermentation system of agricultural biomass, since they can function with a certain degree of oxygen-consuming while breaking down the plant materials, which provides a desirable condition for the subsequent anaerobic

digestion (Kato et al. 2004). However, to the best of our knowledge, no published study has been conducted to explore the aerobic cultivable cellulolytic microbes within the whole GT of ruminants and evaluate their cellulose-degrading potential.

Ujimqin sheep (*Ovis aries*), are widely distributed in pastoral and agricultural areas of Inner Mongolia Autonomous Region in China. In this study, the aerobic cultivable cellulolytic microbes in each GT region of Ujimqin sheep were isolated by using carboxymethyl cellulose (CMC) medium and then identified by 16S rDNA/ITS sequencing. The varied enzyme activities of promising isolates were evaluated during the 10-day incubation with available cellulosic alfalfa hay (*Medicago sativa*). Finally, the changes of plant material surface as well as the bacterial adherent were observed by scanning electron microscopy. This study aimed to enlarge our knowledge of the aerobic cultivable cellulolytic microorganisms along the GT of sheep and provide the essential groundwork for their application in cellulose bioconversion in the future.

Materials and Methods

Ethics statement

All animal experiments were performed in accordance with the Chinese guidelines for animal welfare and experimental protocol, and approved by the Animal Care and Use Committee of Chinese Academy of Sciences. All efforts were made to minimize animal suffering.

Materials and sample collection

Adult Ujimqin sheep (n=5) were collected from Xilingol steppe grassland which is located in northern China and the major vegetation are *Leymus chinensis*, *Agropyron cristatum*, and *Stipa grandis*. The animals used in the present study were 3-year-old, healthy reared sheep and were free-fed while grazing. All animals were aseptically dissected with a sterile scalpel to collect the intestinal content of each GT

region. A total of 50 GT samples (5 sheep $\times$ 10 GT regions) were collected and the whole GT of sheep included stomach (rumen, reticulum, omasum, and abomasum), small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum). The final GT sample used for the cellulolytic microbial isolation was a mixture of same GT region from five sheep individuals. All GT samples were labelled and stored in a portable icebox until being transported to the laboratory.

Microbial enumeration and cellulolytic microbial isolation

The homogenized GT sample (1 ml), which is a composite pooled from the 5 individual sheep samples, was inoculated in 100 ml liquid medium A comprised of CMC 0.2 g, $(\text{NH}_4)_2\text{SO}_4$ 0.2 g, NaCl 0.05 g, K_2HPO_4 0.1 g, $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ 0.05 g, and Congo-red 0.04 g, which was modified based on Dantur et al. (2015). The samples were incubated in a 250 ml conical flask and shaken under 37 °C and 130 rpm for 10 days. To enumerate the total viable count (TVC) of the samples during the 10-day incubation, we collected and then serially diluted the cultures (up to 10^{-10}) on every alternate day followed by spreading each dilution on solid medium A (with additional agar 12 g per liter of solution). The plates were incubated at 37 °C for 72 hours and then enumerated immediately. Three replicates were conducted for each GT sample. Based on the results of enumeration, we collected the enrichment of sheep GT samples in 4th day and diluted samples of abomasum, cecum, colon and rectum to 10^{-8} as well as diluted samples of rumen, reticulum, omasum, duodenum and ileum to 10^{-9} . To further discern the colonies which can excrete cellulases, a second round of incubation were performed. After 3-day incubation, colonies that showed discoloration were considered positive cellulolytic microbes (Teather and Wood 1982) and were isolated for further experiments. After a series of visual screening based on the colony morphologies including color, shape, and margin, only isolates with distinct morphology in each GT region were preserved and performed in the following assays. According to the enzyme activity index (EAI), which

was calculated as the diameter of the clear zone plus the diameter of the colony (Dantur et al. 2015), cellulolytic activities of strains were classified as low (< 10 cm), intermediate (10–20 cm), and high (> 20 cm). The strains that presented high EAI (top 3) were selected and evaluated for the following enzyme activities.

Identification and phylogenetic affiliation of isolates

The DNA from each isolate grown in medium A was extracted by using the PureLink Genomic DNA Mini kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Bacterial universal primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTACCTTGTTACGACTT-3') were used for 16S rDNA gene amplification (Miller et al. 2013), and fungal universal primers (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3') were used for ITS gene amplification (Gardes and Bruns 1993). Each 20 µL reaction mixture contained 4 µL of 5× FastPfu Buffer, 2 units of FastPfu polymerase (TransGen, Beijing, China), 250 mM of dNTP mix, 0.1 µM of each primer, and 10 ng of DNA template. PCR conditions were as followed: initial denaturation step at 95 °C for 3 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min. All PCR reactions were carried out in triplicates and visualized on 1% agarose gel to check the size and purity of the PCR products.

The 16S rDNA/ITS sequencing of each isolate was performed at Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). Sequences were compared with others using Basic Local Alignment Search Tool (BLAST) and the representative microbial sequences from the GenBank database were obtained. For phylogenetic analysis, the alignments of all 16S rDNA/ITS gene sequences were performed with ClustalX (Thompson et al. 1997). The phylogenetic tree was constructed based on a

Neighbor-Joining method with the MEGA version 7.0 software (Saitou and Nei 1987; Kumar et al. 2016), and the bootstrap analysis was inferred from 1000 random replications.

Determination of the cellulolytic enzyme activities

The cellulolytic enzyme activities of promising isolates were determined via total cellulose, endoglucanase, exoglucanase, and β -glucosidase assays. Selected isolates were incubated with cellulosic alfalfa hay (*Medicago sativa*) particles (1% w v⁻¹) in medium B which comprised of (NH₄)₂SO₄ 2 g, NaCl 0.5 g, K₂HPO₄ 1 g, MgSO₄•6H₂O 0.5 g per liter, agitated at 130 rpm, 37 °C for 10 days. Before the inoculation, all the microbial cultures have been normalized to the same concentration (10⁵ CFU ml⁻¹). To optimize the incubation time and monitor the changes in enzyme activity during the 10-day incubation period, we sampled 5 ml of culture on every alternate day. After the collection, enzyme extract was prepared by centrifuging cultures for 10 min at 7000 g and collecting the supernatant. All the enzyme activities were determined based on the method described by Wood and Bhat (1988) with few modifications. Briefly, 1 strip of Whatman No.1 filter paper (1.0 × 6.0 cm), 1% CMC, 1% avicel, and 1% salicin were used as substrates to determine total cellulase, endoglucanase, exoglucanase, and β -glucosidase activity, respectively. The reaction systems were mixed with 1 ml of sodium citrate buffer (0.05 M, pH 4.8) and 1 ml of enzyme solution (appropriately diluted) with different substrates. Then the mixtures were incubated at 50 °C for 0.5 h for both endoglucanase and β -glucosidase activities, while incubated for 1 h and 2 h for total cellulose and exoglucanase activities, respectively. All the reaction was terminated by the addition of 2 ml of dinitrosalicylic acid (DNS) reagent and heating in a boiling water bath for 5 min for color development. Reducing sugars were estimated spectrophotometrically at 540 nm with the DNS method, using glucose as standard. Enzyme activity was recorded as international units per milliliter enzyme extract (IU ml⁻¹), where 1 IU of enzyme activity was defined as the amount

of required enzyme which liberated 1 μmol of glucose equivalent per min under the assay conditions. All enzyme assays were performed in triplicate in this study.

Scanning electron microscopy (SEM)

Samples of alfalfa hay particles before and after *in vitro* 10-day incubation with selected strains were harvested from medium B. The plant fragments (~1 mm) were prepared and the bacterial cells were fixed by immersion into 2.5% glutaraldehyde at 4°C overnight. After the removal of glutaraldehyde, the plant fragments were rinsed with deionized water completely after three times of immersion which accounts for 5, 6, and 7 min, respectively. Then the plant fragments were stepwise dehydrated in 50%, 70%, 85%, 95%, and 100% concentrations of ethanol for 15 min of each immersion, followed by final dehydration in 100% ethanol with three consecutive duplicates. These fragments were immersed in absolute ethanol and critical point dried subsequently, to preserve the surface structure during microscopic observation. Finally, the dried samples were transferred on SEM stubs and covered with a layer of gold. The surface morphology of the plant fragments as well as the adhered-microbes was observed by scanning electron microscope SU8010 (Hitachi High-Technologies Co., Japan).

Availability of data

The datasets generated during the current study are available in the GenBank with accession numbers MT043867–MT043874 and MT043876–MT043889, respectively.

Results

Enrichment, isolation, and screening for cellulolytic microorganisms

The dynamic change of TVC during the 10-day enrichment was monitored on every alternate day. As shown in Fig. 1, TVC before the incubation differed among GT regions, with the highest TVC observed in the rumen mixture (9.80×10^7 CFU/ml), followed by rectum mixture (4.10×10^7 CFU/ml) and colon

mixture (1.67×10^7 CFU/ml), whereas the lowest microbial load was observed in the jejunum mixture (not detected). During the 10-day incubation, the monitoring of TVC during the incubation showed that TVC has been increased for the first 4 days and then dropped in the following days. The highest microbial load during the incubation was observed in the omasum mixture (7.68×10^{10} CFU/ml).

To preliminarily isolate the strains with cellulose-degrading ability, Congo-red stained agar plates on CMC were used to screen out the CMCase-secreting isolates (Fig. 2). After an appropriate dilution followed by a second round 3-day incubation, a total of 67 isolates with discoloration ability were collected from enrichment cultures of all sheep GT regions. Via several rounds of strain purification, we filtered out colonies with similar size, color, and morphology within each GT region, and obtained 22 isolates with visible clearance zones surrounding the colony, which were selected to conduct the following taxonomy analysis and evaluation for cellulose biodegradation. Of the 22 cellulolytic isolates, a maximum of 7 strains was isolated from the rectum mixture, followed by 4 strains isolated from the rumen mixture, and 3 strains isolated from the ileum mixture (Table 1).

Phylogenetic analysis of microbial isolates and their diversity in different sheep GT

The 16S rDNA/ITS genes of the 22 isolates were amplified and sequenced. The generated sequences were compared with Genbank database using BLAST analysis. The closest matches with the highest score were shown for each isolate (Table 2). Except for one unknown bacteria (IL147), the rest of the isolations belonged to three bacterial phyla, i.e., Actinobacteria (9 isolates), Proteobacteria (2 isolates), and Firmicutes (2 isolates), as well as one fungal phylum Ascomycota (8 isolates). The phylogenetic relationships of the cellulolytic isolates and the representative strains from the GenBank were further analyzed. As shown in Fig. 3, these isolates revealed their close related matches to 9 different genera, 3 of the Actinobacteria phylum (*Brachybacterium*, *Sanguibacter*, and *Streptomyces*), 2 of Firmicutes

phylum (*Lactobacillus* and *Bacillus*), 2 of Proteobacteria phylum (*Rhizobium* and *Pseudomonas*), and 2 of the Ascomycota phylum (*Fusarium* and *Aspergillus*). In *Streptomyces* branch, the isolates RE1610, RU231, and OM13 were closely related to *Streptomyces hydrogenans*, whereas RE1410, RE1710, and RE1910 were more related to *Streptomyces microflavus*. IL57 was placed in a separate branch of the tree and showed only 98% of identity with *Streptomyces* sp. MM106 16S rRNA gene sequence (Table 2). Besides, IL147 was grouped in the same branch with uncultured bacterium (JQ798433.1) and *Rhizobium* sp. (KY630724.1) as shown in Fig. 3, and the BLAST results showed that the *Rhizobium* sp. strain WKFF29 is the best match of strain IL147 with a well-described bacteria in the current GenBank database, with the score of 2377 (the third-highest score after the first two “uncultured bacterium”) and identity of 99%.

The distribution of isolates from different GT regions was shown in Supplementary Fig. S1. At the genus level, the aerobic cultivable cellulolytic microbes isolated from the stomach and large intestines were more diverse than those isolated from the small intestine, which accounted for 56% (stomach), 67% (large intestine), and 33% (small intestine) of the total identified genera within the entire GT, respectively. Among the cellulolytic isolates, members of *Aspergillus* and *Streptomyces* were dominated in the entire GT, with 64% of the isolates affiliated with these two genera; and they also widely distributed along the whole GT, which were detected in 7 and 4 of entire GT regions, respectively.

Screening for cellulolytic activity

Agar medium with Congo-red as an indicator provides a rapid and primary method for determining the cellulolytic activity of the isolates. The cellulolytic activities of the isolates were measured for the 22 aerobic cultivable cellulolytic isolates using soluble substrates CMC. After 3 days of incubation, the cellulolytic activities of the isolates were evaluated for the presence of a clearance zone around each

colony. Based on the EAI score, there were 4, 10, and 8 isolates that presented high, intermediate, and low cellulolytic activities, respectively (Table 1). The cellulolytic activity of the isolates from the rectum and rumen were comparably high, with three and one strains exhibiting considerable cellulose degradation activity (EAI>20; Table 2), respectively. Especially, it is worthy noted that most isolates (86%) from the rectum exhibited high or intermediate activity; while isolates from other GT regions (except of rectum), generally presented an intermediate or low activity comparatively (excluding of one isolate from rumen). This demonstrated that multiple cellulolytic microbes colonized in the sheep rectum possess a competent ability to produce CMCase under aerobic conditions. Of all the isolates, the strain of *Pseudomonas* sp. (RU61) generated the largest clearance zone (diameter=33.00 cm) after 3 days of growth. Strains of RE2510 belonging to *Bacillus subtilis* and RE1410 belonging to *Streptomyces* also showed large areas of discoloration with a diameter of 27.00 cm and 26.17 cm on average, respectively. Owing to their superior cellulolytic activities, isolations encoded as *Pseudomonas* sp. RU61, *Streptomyces* sp. RE1410 and *Bacillus subtilis* RE2510 were selected for further various cellulolytic enzyme assays.

Determination of enzyme activity

The enzyme activity assays were performed to further investigate the cellulolytic potential of the selected strains. For both *Streptomyces* sp. RE1410 and *B. subtilis* RE2510, the total cellulose activities tended to be increased during the whole 10-day incubation. By contrast, *Pseudomonas* sp. RU61 showed the highest total cellulose activity on the 6th day and then decreased gradually (Fig. 4A). After 10-day incubation, both *B. subtilis* RE2510 and *Streptomyces* sp. RE1410 showed high total cellulose activities (over 0.14 IU ml⁻¹ in average). For the endoglucanase activity, all selected isolates increased during 10-day incubation, especially with a rapid increase after 4 days of incubation (Fig. 4B). Among the three

strains, the maximum endoglucanase activity after 10 days of incubation was observed in *B. subtilis* RE2510 (0.1478 ± 0.0014 IU ml⁻¹). As for exoglucanase activity, although the activities of the selected strains presented a general increasing trend during 10-day incubation, the exoglucanase activity of them peaked in different incubation days (Fig. 4C). *Pseudomonas* sp. RU61 achieved its highest exoglucanase activity (0.2200 ± 0.0103 IU ml⁻¹) after 6 days of incubation, while *B. subtilis* RE2510 peaked after 8 days of incubation (0.2097 ± 0.0166 IU ml⁻¹) and *Streptomyces* sp. RE1410 peaked after 10 days of incubation (0.1725 ± 0.0013 IU ml⁻¹). After 10-day incubation, the maximum exoglucanase activity was found in *B. subtilis* RE2510 (0.1735 ± 0.0012 IU ml⁻¹). The change of β -glucosidase activity during the incubation varied among selected strains. Both *B. subtilis* RE2510 and *Pseudomonas* sp. RU61 showed rapid increased β -glucosidase activity before the first 6 days of the incubation and then increased smoothly, while the *Streptomyces* sp. RE1410 peaked after the 6th day and then decreased till the incubation ended. Notably, the maximum β -glucosidase activity after 10-day incubation was found in *B. subtilis* RE2510 (0.3817 ± 0.0031 IU ml⁻¹, Fig. 4D), which is approximately 1.5 times higher than those in *Pseudomonas* sp. RU61 and 3 times higher than those in *Streptomyces* sp. RE1410. In sum, after 10-day incubation, *B. subtilis* RE2510 exhibited the highest endoglucanase, exoglucanase activities, and β -glucosidase activity when grown on alfalfa hay, indicating that *B. subtilis* RE2510 can efficiently break β -1,4-linkages, cellulose chains, and metabolize cellobiose units under the current culture conditions (Beguin and Aubert 1994).

Morphology and bacterial adhesion to the cellulosic substance

To verify that *B. subtilis* RE2510 isolated from the GT of sheep can attach and degrade the rigid structure of the cellulosic substrates, SEM was performed on alfalfa hay which was incubated with *B. subtilis* RE2510. As shown in Fig. 5, the microstructure of the plant substrate was changed notably after the 10-

day incubation. The tissue organization of the alfalfa hay cell wall was smooth and intact before incubation (Fig. 5a), but changes were detected in vascular bundles and parenchyma cells in alfalfa hay *a* after its incubation with *B. subtilis* RE2510 (Fig. 5b). The remaining substrate samples showed a high fractured and porous structure with a large amount of typical round-shaped pores compared with the original grass substrate. In addition, SEM observations also intuitively showed that a number of bacteria adhered to the substrate after incubation, with the *B. subtilis* RE2510 attaches as single rod-shaped bacterial cells (Fig. 5c).

Discussion

GT of ruminants has been considered as an efficient bioreactor to convert plant materials to valuable products and thus attract special attention from researchers for detecting promising cellulolytic microbes. Although efforts of anaerobic cellulolytic microbes within ruminant GT have been accumulated to date, the knowledge of aerobic cultivable microbes with cellulolytic activities in GT is still largely unexplored. In this study, we obtained 22 aerobic or facultative aerobic cellulose-degrading microorganisms from different GT regions of Ujimqin sheep (*Ovis aries*), wherein most of the isolates were obtained from the large intestine and stomach compared with the small intestine (Table 1). The limited cellulolytic microbes isolated from the small intestine might be associated with the intrinsic lower bacterial diversity in sheep (Wang et al. 2017; Zeng et al. 2017). Furthermore, previous carbohydrate-active enzyme analysis found that the glycoside hydrolases (GHs) and polysaccharide lyases (PLs) in the stomach and large intestine of sheep were much higher than those in the small intestine (Al-Masaudi et al. 2017), but further quantitative analysis of these cellulolytic microorganisms in sheep GT is still required.

The significant outcome of the present study is the isolation and identification of a set of aerobic

cellulolytic microbes from the GT of sheep. The aerobic cultivable cellulolytic members in the sheep GT isolated here were dominated by members of Actinobacteria, Firmicutes, Proteobacteria, and Ascomycota, and these phylum have also been reported as common GT colonizers in sheep based on the high-throughput sequencing analysis in previous literature (Zhang et al. 2018; Yang et al. 2019). The refined alignment showed that the isolated aerobic cellulolytic microbes were identified as 9 different genera (Fig. 3). Among these genera, most of them have been characterized as common GT colonizers of several sheep species, such as small tailed Han sheep (Wang et al. 2017), Mongolian sheep (Zeng et al. 2017) and Hu sheep (Xie et al. 2018), etc. Although these microbes have been detected via molecular biotechnology in previous work (Al-Masaudi et al. 2017; Xie et al. 2018; Yang et al. 2019), our study further confirms their cultivable possibility and cellulolytic potential in aerobic environments. Notably, members of *Fusarium*, *Aspergillus*, and *Rhizobium* were first isolated from Ujimqin sheep GT under aerobic conditions and also present clear cellulolytic activities in this study. In contrast, the microbes which have been reported in previous metagenomic work, but were not detected here, might be associated with the requirements for strict anaerobic conditions (e.g., species belonging to *Ruminococcus* and *Fibrobacter*), unknown cellulolytic potential, or unfavorable culture media, etc. Overall, these results revealed several aerobic cultivable microbes with cellulolytic activities within sheep GT and more cultivatable efforts regarding modification of the *in vitro* conditions may facilitate the exploration of more aerobic cellulolytic microbes from ruminant GT.

Previous literature has demonstrated that Actinobacteria contains many well-known cellulose degraders and nearly half of the strains isolated from the sheep GT here belonged to the phylum Actinobacteria (Fig. 3). Among them, *Streptomyces* spp. have been isolated from sheep feces previously, but their cellulolytic activity was rarely to be verified. In fact, genomic analyses have

demonstrated that *Streptomyces* genomes contain abundant Carbohydrate-Active Enzymes (CAZy) and accessory protein (Berlemont and Martiny 2013), which indicated the potential role of *Streptomyces* in the deconstruction of plant biomass. *Streptomyces* sp. RE1410 isolated from the rectum of sheep here, presented a comparably higher cellulolytic activity than those of *Streptomyces* strains isolated from soil or marine sediments in previous reports (Table S1), suggesting the significant role of *Streptomyces* species in the degradation of dietary fiber in sheep GT. In addition, isolate affiliated with *Sanguibacter* (RU111) and *Brachybacterium* (RET82), both of which also belonged to phylum Actinobacteria, showed an intermediate and low cellulolytic activity, respectively. Although strains belonging to these genera have been reported as potential cellulose degraders (Cook et al. 2007; Zhang et al. 2007), the modest cellulolytic activity of these bacteria here suggested that their major function in sheep stomach may not be limited to cellulose degradation. Nevertheless, further analysis to discern the major role of these microorganisms in sheep GT is still required.

Members of genus *Lactobacillus* are well-known lactic acid bacteria (LAB) as their extensive applications in foods and probiotics, but knowledge regarding its role in cellulose-degrading mainly focused on the construction of recombinant cellulolytic LAB strains (Mazzoli et al. 2014). In this study, a natural cellulolytic strain *Lactobacillus* sp. (RE2210), which was isolated from sheep rectum, exhibited an intermediate level of cellulolytic activities (Table 2). A recent study also revealed the presence of gene coding for a cellulose-degrading enzyme in four *Lactobacillus* spp. strains based on comparative genome analysis, which confirm the possibility of the cellulolytic potential of *Lactobacillus* spp. from a genetic perspective (Wuyts et al. 2019). As shown in previous work, a combination of cellulose-decomposing bacteria and LAB, or engineered LAB strains containing cellulose gene (such as *bgl1*, *cbh2* and *egl3*) could enhance the conversion rate of cellulolytic

substrates during the ensiling (Zhang et al. 2019; Liu et al. 2020). The natural cellulolytic LAB strain observed here not only broadens our knowledge of natural cellulolytic degrading abilities in *Lactobacillus* spp., but also provided new insights into using natural cellulolytic LAB to promote the bioconversion of cellulosic biomass.

Further cellulolytic enzyme activity of the CMC-degrading isolates indicated that strain *B. subtilis* RE2510 possess various extracellular enzyme activities and have significant potential to degrade lignocellulosic substrate alfalfa hay (Fig. 4-5). It has been widely reported that genus *Bacillus* contained numerous aerobic or facultative aerobic cellulose-degrading bacteria, which can be isolated from soil or other waste materials with high cellulose content (Tondo et al. 1998; Su et al. 2006). Also, *B. subtilis* can express multi-cellulase containing minicellulosomes on its surface to enhance the degradation of lignocellulosic biomass (Liu et al. 2012; You et al. 2012). To date, *B. subtilis* have been demonstrated as one of the efficient feed additives for ruminant due to their multiple enzyme-producing properties and high protein content (Zayed et al. 2020), and using cellulose-degrading bacteria isolated from ruminant GT as feed additive may improve the silage quality while minimizing the adverse effects of ruminants on the microbial additives during the feeding and digestion process. Furthermore, *Bacillus* strains present a higher tolerance to the harsh environment owing to their spore-forming characteristic, which could provide them with a greater advantage over other strains under challenging conditions such as variable temperatures and slow growth on cellulosic substrates.

Conclusion

The current study presents the composition of aerobic cultivable cellulolytic microorganisms in GT tracts of Ujimqin sheep (*Ovis aries*). Twenty-two isolates were identified by 16S rDNA or ITS sequencing, and phylogenetic analysis revealed that all strains belonged to four phyla, i.e.,

Actinobacteria, Firmicutes, Proteobacteria, and Ascomycota. Among them, *B. subtilis* RE2510 showed the highest endoglucanase, exoglucanase, and β -glucosidase induced on alfalfa hay after 10-day incubation. Moreover, this strain also exhibited significant damage to the cellulose structure of alfalfa hay after the incubation, which indicates *B. subtilis* RE2510 could be a potential candidate for biodegradation of plant lignocellulosic substrate. Considering the good cellulolytic activity of *B. subtilis* RE2510 isolated from ruminant GT, further studies are required to optimize fiber digestibility with this strain and to evaluate the effect of this microbial additive on the feed intake of ruminants.

Acknowledgments

This work was supported by the Science and Technology Service Network Initiative Project of the Chinese Academy of Sciences (no. KFJ-STIS-QYZX-112). We thank Mr. Jianjun Chen and his colleague for their help with sample collection.

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Figure legends

Fig. 1 Total viable count of aerobic cultivable microorganisms from different GT of sheep enriched on CMC. CFU, colony forming units.

Fig. 2 Examples of Congo-red staining of aerobic cultivable cellulolytic isolates from sheep GT. Strains were designated as RE and RU represented isolation from rectum and rumen, respectively. Based on the 16S rDNA sequencing, RE2510 was identified as *Bacillus subtilis*, RE1410 was identified as *Streptomyces* sp., RU61 was identified as *Pseudomonas* sp., RU111 was identified as *Sanguibacter soli*, and RU231 was identified as *Streptomyces* sp.

Fig. 3 Phylogenetic tree based on analysis of isolated aerobic cultivable cellulolytic microbes in this study and their close representatives in GenBank database. The tree was constructed by using the Neighbor Joining method with Kimura-2 parameter distance estimates and the numbers at nodes represent bootstrap values (1000 trials). The cellulolytic activity of each isolate is recorded in parenthesis (+, low level; ++, intermediate level; and +++, high level).

Fig. 4 Cellulolytic enzyme activities of tested aerobic cultivable isolates incubated with alfalfa hay during the 10-day incubation. Values represent means $\pm$ S.D (n=3).

Fig. 5 Scanning electron microscopy (SEM) images of alfalfa hay samples before (a) and after (b) incubation, and bacterial attachment of *Bacillus subtilis* RE2510 (c) to the substrate.

Table 1 Distribution of aerobic cultivable cellulolytic isolates from different GT regions of Ujimqin sheep (*Ovis aries*).

Gut region		Initial number of isolates	Final number of isolates	Number showing highest cellulolytic activity	Number showing medium cellulolytic activity	Number showing lowest cellulolytic activity
Stomach	Rumen	20	4	1	3	0
	Reticulum	7	2	0	0	2
	Omasum	1	1	0	1	0
	Abomasum	9	1	0	0	1
Small intestine	Duodenum	0	0	0	0	0
	Jejunum	0	0	0	0	0
	Ileum	4	3	0	1	2
Large intestine	Cecum	7	2	0	1	1
	Colon	3	2	0	1	1
	Rectum	16	7	3	3	1
Total		67	22	4	10	8

The values in the column ‘initial number of isolates’ mean the number of the isolates with discoloration before the morphology filtering. The values in the column ‘final number of isolates’ means that the final number of the discolored strains after the filtering of those isolates with similar size, color and morphology within each GT region.

Table 2 Molecular identification of the aerobic cultivable cellulolytic strains isolated from sheep GT.

	Source	Strain name	Accession of closest match organism	GenBank description	Score	Identity (%)	Coverage (%)	Phylum	CMC (EAI)	Accession No. of the isolates
1	Rumen	RU111	MN646988.1	<i>Sanguibacter soli</i> strain CBA7525	2584	99%	99%	Actinobacteria	14.00±0.00	MT043880
2	Rumen	RU21	MN310533.1	<i>Aspergillus versicolor</i> strain	1059	100%	99%	Ascomycota	19.33±3.06	MT043870
3	Rumen	RU61	MN810183.1	<i>Pseudomonas</i> sp. strain BIS1097	2619	99%	99%	Proteobacteria	33.00±0.00	MT043878
4	Rumen	RU231	MG893105.1	<i>Streptomyces</i> sp. strain JSM 1684074	2610	99%	99%	Actinobacteria	18.33±0.58	MT043888
5	Reticulum	RET172	MN310533.1	<i>Aspergillus versicolor</i> strain XWF32-16	1055	100%	99%	Ascomycota	9.83±1.61	MT043869
6	Reticulum	RET82	CP060496.1	<i>Brachybacterium</i> sp. Z12	2558	99%	98%	Actinobacteria	7.83±2.47	MT043879
7	Omasum	OM13	MH549256.1	<i>Streptomyces</i> sp. clone ZYP16	2595	99%	98%	Actinobacteria	19.67±0.58	MT043876
8	Abomasum	AB224	MN310533.1	<i>Aspergillus versicolor</i> strain XWF32-16	1057	100%	99%	Ascomycota	8.83±1.04	MT043873
9	Ileum	IL147	JQ798433.1	Uncultured bacterium clone sdm63 16S ribosomal RNA gene, partial sequence	2394	98%	99%	Unknown Bacteria	4.50±1.32	MT043882
10	Ileum	IL57	KU714871.1	<i>Streptomyces</i> sp. MM106	2582	99%	98%	Actinobacteria	7.33±1.15	MT043877
11	Ileum	IL67	MN310533.1	<i>Aspergillus versicolor</i> strain XWF32-16	1057	100%	99%	Ascomycota	10.50±1.32	MT043867
12	Cecum	CE158	MN310533.1	<i>Aspergillus versicolor</i> strain XWF32-16	1057	100%	99%	Ascomycota	8.83±2.47	MT043872

13	Cecum	CE67	CP036186.1	<i>Pseudomonas stutzeri</i> strain ATCC 14405	2614	99%	99%	Proteobacteria	18.67±0.58	MT043881
14	Colon	CO119	AY188923.1	<i>Fusarium tricinctum</i>	1044	100%	99%	Ascomycota	6.67±1.53	MT043871
15	Colon	CO79	MN310533.1	<i>Aspergillus versicolor</i> strain XWF32-16	1038	99%	99%	Ascomycota	19.33±3.51	MT043868
16	Rectum	RE1410	CP029342.1	<i>Streptomyces</i> sp. SM18	2593	99%	99%	Actinobacteria	26.17±1.04	MT043883
17	Rectum	RE1610	MG893105.1	<i>Streptomyces</i> sp. strain JSM 1684074	2601	99%	99%	Actinobacteria	20.33±1.53	MT043884
18	Rectum	RE1710	MG833348.1	<i>Streptomyces</i> sp. strain LJL22	2590	99%	98%	Actinobacteria	10.50±0.50	MT043885
19	Rectum	RE1910	EU273548.1	<i>Streptomyces microflavus</i> isolate XSD-113	2604	99%	99%	Actinobacteria	10.67±0.58	MT043886
20	Rectum	RE2210	MT510485.1	<i>Lactobacillus fermentum</i> strain 5958	2699	99%	99%	Firmicutes	12.83±1.04	MT043887
21	Rectum	RE2410	MN310533.1	<i>Aspergillus versicolor</i> strain XWF32-16	1055	100%	99%	Ascomycota	4.00±1.00	MT043874
22	Rectum	RE2510	MT641205.1	<i>Bacillus subtilis</i> strain soilG2B	2621	100%	100%	Firmicutes	27.00±1.00	MT043889

CMC, carboxymethyl cellulose; EAI, enzymatic activity index. The closest organism is identified as the best match organisms as shown in BLAST reports, which is sorted by the default E value. Numbers in the column of EAI represent diameter of halo in centimeters. Strains were let to grow for 3-7 days at 37 °C before transfer to a new plate for the EAI measure. The values in the column of score represent the alignment scores between the query sequence and the database segments while those in column of identity represent the number of matching bases over the number of alignment columns.

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214x178mm (300 x 300 DPI)

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112x24mm (600 x 600 DPI)

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149x122mm (600 x 600 DPI)

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