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ORIGINAL ARTICLE

Screening and evaluation of the ruminal cellulolytic bacteria and their potential application as probiotics

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ABSTRACT

Background and Objectives: Rumen microbiologists are looking for new probiotics to improve the digestibility of livestock diets. This study intended to screen and evaluate the ruminal cellulolytic bacteria (CBs) and their potential application as probiotics.

Materials and Methods: Microbial culture and molecular techniques performed to isolate CBs from the rumen of camels, deer and rams. Their antibacterial and antibiogram tests were done using disc diffusion method. Their potential to degrade cellulose, starch, tannin and protein were investigated using clear zone halo, and spectrophotometric techniques. Bilious, saline, and acidic broth media were used to study the resistance of isolates in intestinal conditions.

Results: The phylogenetic analysis revealed that the strains belonged to Firmicutes and Proteobacteria phyla, *Citrobacter murliniae*, *Ornithinibacillus bavariensis*, *C. braakii*, and *Bacillus subtilis*. The highest cellulase (CAS) activity was recorded by *C. murliniae* Dez wildlife13A (2.98 UmL^{-1}), whereas *C. braakii* Loot desert 111A (1.14 Uml^{-1}) was produced the lowest enzyme. The isolates were highly resistant to synthetic conditions of intestine (pH 2.5-3.5, bile 0.3-2%), as well as tolerated higher concentrations of NaCl (up to 10%). They effectively inhibited standard pathogen strains, and showed sensitivity to the used antibiotics.

Conclusion: This study reports the cellulolytic *O. bavariensis* Tabbas desert 32A for the first time from the rumen, which will have potential biotechnological applications.

Keywords: Camelus; Cellulase; Deer; *Ornithinibacillus bavariensis*; Probiotic

INTRODUCTION

The cellulosic polymers have the largest share in plants cell walls, which are homogenous macromolecule, fibrous, and naturally occurring polymers. In terms of its organic nature, these polymers are the most abundant and agriculture untapped renewable sources (1). Every year, a large volume of cellulosic

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biomass (CBM) is produced throughout the world, with major part being incinerated in the fields after harvesting the crops, adding to the greenhouse gas emissions (2). Due to highly stable and complex structure, the nutritional and biotechnological applications of cellulose are difficult. However, efficient bioconversion of cellulose could yield value-added nutrients, as seen in ruminants. These animals can convert CBM into i.e., meat and milk that is achieved through symbiotic association with gut microbes particularly bacteria (2). Hence, the specialized foregut found in buffalo, cows, sheep, goats, camel, and deer is considered as a suitable site of cellulose degradation (3-7). Rumen microbiota is composed of different communities of bacteria, protozoa, archaea and fungi, which efficiently convert cellulose to volatile fatty acids as an energy source for host animal (3-5). The symbiotic microbes inherit an arsenal of enzymatic systems called cellulases that are responsible for breakdown of cellulose polymer into simple sugars (4). The CASs are inducible enzymes, which can degrade CBM into fermentable mono or oligosaccharides and energy (3-5). The effective hydrolysis of cellulose involves the synergistic activity of three enzymes, viz, exo-endoglucanase, cellobiohydrolase, and β-glucosidase that together constitute CAS complex. Cellulose-degrading enzymes are essential for use in different industries such as textile, biofuels, paper, detergents, food, and animal feed (3-5). These enzymes are necessary need for the industry due to their modular architecture, substrate-specificity and other biochemical properties (8). Recently, cellulase-producing bacteria isolated from the animal's gut have been formulated as probiotics to replace antibiotics, for example *Lactobacillus, Bifidobacteria* and *Escherichia genera* (9, 10). Furthermore, enzymatic byproducts can be used by probiotic microbes as prebiotic components. These live microbes will probably help to improve the digestion of highly cellulosic forages (11), and when administered in sufficient doses can enhance host health indicators (10). Therefore, these microorganisms should be resistant to acidic pH, and toxic bilious acids in the digestive system besides inhibiting the pathogens (9). These bacteria can maintain gut in a stable pH, while enhance anaerobic digestion and supply of nutrients to the host, therefore depicting probiotic capabilities (9-13). Predominantly found CBs genera comprise *Ruminococcus, Fibrobacter, Megasphaera, Streptococcus, Alicyclobacillus, cellulosilyticus, Anaerobacterium, chartisolvens, Escherichia, Chytridiomycetes fungi*, and methanogens, while about 70% of bacterial population remains undiscovered (3-9). These microbes have been isolated from the sources other than the rumen of Urial rams (URs), Fallow deer (FDs), and Balochi camels (BCs). They are known as browsing animals feed on wooden forages rich in dicots, cellulose, and phenol. Owing to the rare studies on the ruminal probiotics, the current *in vitro* study was conducted to screen and evaluate the rumen bacteria towards cellulose degradation and their potential application as probiotics. Further, the isolates were characterized and identified through molecular sequencing approaches. Based on the available literature, the probiotic potential of the *O. bavariensis* Tabbas desert 32A identified in the rumen of URs was proposed for the first time in this research. We believe that these findings may open new avenues for the industrial use of these isolates in the production of commercial enzymes and animal feed additives.

MATERIALS AND METHODS

Animals and their habitats. The URs (*Ovis vignei*) and BCs (*Camelus dromedarius*) were lived in the desert areas of Birjand, Tabas, and Nehbandan (South Khorasan province, Iran), while the FDs (*Dama dama*) had reared in the Dez Wildlife Park (Kupitte nearly 10 Km from the Dezful city, Khuzestan province, Iran). The forges consumed by the animals were included *Olea europaea, Pistacia terebinthus, Conocarpus erectus, Alhagi persarum, Prunus scoparia, Ferula assa-foetida, Zygophyllum eurypterum, Ficus carica, Acacia nummulorii, Astragalus* sp., *Albizia lebbeck*, and *Eucalyptus microtheca*, etc. These animals had free access to forage and water. The rumen sampling was done accorded to the animal standard guidelines (14).

Sample collection and preparation. All the experiments were carried out in the biotechnology and microbiology laboratories (Animal Science Department, Faculty of Agriculture, University of Birjand, as well as in the Department of Biology, Faculty of Science, Shahid Chamran University of Ahvaz, Iran) during the years 2021 to 2022. The rumen contents (RCs) were collected from the five years old males of BCs (n=3), and two years old 4 males' of URs (n=4).

However, in case of the FDs, the RCs were sampled from the rumen of 2 females and 2 males (2 years old, n=4) through a stomach tube and a vacuum pump. The RCs of BCs was immediately taken from the rumen of slaughtered animals. The RCs samples aseptically collected in sterile bottles flushing with 5% CO_2. Under standard conditions, RCs were transferred to the microbiology laboratory, and filtered through double-layered sterile muslin cloth.

Screening of cellulolytic bacteria. First, 10 mL of rumen fluid was added in the flasks containing 100 mL of the sterile nutrient broth (NB) media. The flasks were transported to an incubator (39°C, according to rumen temperature as a source of bacteria isolation) for 48 h. After incubation, 10 mL of the enriched bacterial suspension was reinoculated into fresh NB, and incubated for 48 h. Subsequently, samples were collected in small aliquots (~2 mL), and 1 mL of each sample was homogenized for 5 min, then diluted in 9 mL of sterilized physiological solution (10^{-1} to 10^{-9}). From each tube, 100 µL was spread on nutrient agar (NA) media in duplicate sets, and incubated (39°C, 48 h). After that, the properly grown bacteria picked and purified on NB plates through repeated streaking and sub-culturing methods (5, 6).

The purified colonies were inoculated into 9 mL of freshly prepared NB media containing 1% carboxymethyl cellulose (CMC), and incubated (39°C, 48h). In addition, a loopful of each bacterium were grown on 1% CMC-NA plates at 39 °C for 48 h. Herein, staining solution (0.1 g of Congo red powder in 100 mL sterilized water) was used for 15 min, then stained-agar plates washed with 1 M NaCl solution for 5 min. The clear halo around the bacterial colonies was indicated cellulose hydrolysis. For each isolate, the hydrolysis capacity (HC) wad determined as the ratio of clear zone to the colony diameter (16). The cellulase positive isolates were preserved as 20% glycerol stocks and selected for further studies.

Morphological and biochemical characterization. The morphology of isolates including array, size, shape and endospore were studied using light microscopy. Strains were stained using crystal violet and safranin dyes by Gram method (15). They were further characterized via growth on MacConkey Agar, Bile Esculin Agar, Eosin Methylene Blue (EMB), and Citrate Agar, using sold media plates. The Sulfide Indole Motility test (SIM) and reaction to the KOH, oxidase and catalase reagents were done by following the methods of Cappuccino & Sherman (16), using *Escherichia coli* as a reference.

Cellulase activity assay. The superior cellulolytic strains were preferred for CAS characterization. The isolates (OD_{600}= 0.6) were inoculated into sterilized NB tubes supplemented with 1% CMC as substrate, and allowed to grow in a shaker incubator at 150 rpm (39°C, 48 h). One mL of bacterial suspension was centrifuged to obtain crude enzyme extract (3000×g, 20 min). For CAS activity, 0.2 mL of the bacterial supernatant were inoculated in 1.8 mL of CMC solution (1% in 0.05 mM PBS, pH 5.5), and placed at an aerobic incubator (39°C for 30 min). Thereafter, 1 mL of 3, 5, dinitrosalicylic acid reagent (DNSA) was added to the mixture of reaction to stop the product formation (17), and the solution was seethed for 15 min. Herein, an ice bath was used to cool the mixture. The reaction mixture was read at 620 nm by spectrophotometry. The CAS activity of the strains was measured in international units (IU), which is equal to the volume of enzyme required to release reduced sugar, 1 µmol per min (17).

Enzymatic profile of the potential isolates. The enzyme ability of the bacteria to decompose starch, protein, and tannin was evaluated by measuring a clear zone diameter (18, 19). The proteolytic activity (PA) of the potential bacteria was determined through 1% bovine serum albumin (BSA)-NA plates. A loop full of fresh bacterial culture (OD of 1) was spread on BSA-NA plates, and allowed to incubate (39°C, 48 h). The NA plates flooded with 1% glacial acetic acid were examined for translucent halos around the inoculation point (19). For tannase activity (TA), the strains were inoculated on 1% tannic acid-NA plates (pH 7) and incubated (39°C, 48 h). The Petri dishes were immersed in a 1 M solution of $FeCl_3$ for 20 min and evaluated carefully for a clear area around the colonies. The amylase activity (AA) of the isolates were studied using 1% starch-NA medium. In a straight line, the strains were cultured on starch agar medium, and allowed to incubate (39°C, 48 h). Subsequently, the agar media were stained with crystalline iodine to observe the luminous zones around bacteria, indicating hydrolysis of starch as a source of carbon (18).

Growth in different conditions. These CBs were

investigated for tolerance to bile salt, sodium chloride and low pH through NB media supplemented with different concentrations of bile salt (0.3% to 2%), and NaCl (2%-10%). The optimum temperature for bacterial growth was further studied, ranging from 27-45°C. To determine suitable pH for CAS activity, NB media was prepared with pH of 2.5 to 8 with the help of NaOH or HCl solutions prior to inoculation. Subsequently, each tube was inoculated with a freshly cultured isolate and incubated (39°C, 48 h). Then, the bacterial growth in the experimental tubes was compared to the control having neutral pH 7.0 (20).

Antipathogenic potential. The antipathogenic potential of the strains was examined using disk diffusion method (21). The standard pathogen bacteria including *Escherichia coli* (ATCC25299), *Staphylococcus aureus* (ATCC25923), *B. subtilis* (ATCC6633), and *Pseudomonas aeruginosa* (ATCC27853) were cultured in broth media with concentration of 0.5 MacFarland (Brain Heart Infusion, BHI). To obtain cell free extract, 200 µL of bacterial suspension (10^7 CFU/mL) was centrifuged at 13000 g for 1 min, then passed through 0.22 mm syringe filters. The pathogen strains were spread on Muller Hinton agar (MHA) plates, then paper-blank discs were smeared with 100 µL of filtered supernatant and put on the plates. The inoculated agar media were placed in an incubator (39°C, 24 h). The 100 µL of sterile milli-Q water was used as the control, and this experiment was repeated more than 3 times. The inhibition zone around the impregnated paper discs was confirmed as antibacterial effect (mm).

Antibiogram reaction. The sensitivity of the strains to antibiotic was investigated through disk diffusion technique (22), with slight modifications. Precisely, the overnight bacterial culture was prepared, and spread uniformly with a sterile cotton-swab on MHA plates. The antibiotic disks containing rifampin (5µg), kanamycin (30µg), Furazolidone (100µg) and amoxicillin (25µg) were pasted on MHA plates using sterile forceps. These plates having antibiotic disks were incubated (39ºC, 24 h), and zone of inhibition detected using a standard and scientific ruler (mm). The obtained results were reported as susceptible, intermediate or resistant strains (https://clsi.org). The control set used for this test included the bacterial cultures devoid of antibiotic.

Phylogenetic identification. The strains were identified through 16S rRNA gene sequencing. The bacterial DNA were obtained using the boiling method (23), and eluted in sterile double distilled water (100 µL), then extracted DNA was quantitatively evaluated by spectrophotometry (>1.8, at 260/280 nm). The purity of the DNA products was estimated through 0.8% agarose gel electrophoresis. Subsequently, the gene fragments were amplified by polymerase chain reaction (PCR) in a thermocycler (Germany, Eppendorf Company). The PCR reaction was composed initial denaturation (92°C for 120 s), which followed by a 36-cycles of denaturation at 92°C for 60 s, annealing at 48°C for 30 s, elongation at 72°C for 120 s, and final extension at 72°C for 370 s. The volume of PCR reaction was 25 µL consisted of 1 µL of forward primer (27F-5′AGAGTTTGATCCTGGCTCAG-3′), 1 µL of reverse primer (1492R 5′-ACGGGCGGTGTGTTC-3′), 12.5 µL of 2X master mix (Parstus, Iran), 9.5 µL of RNAse free water, and 1 µL of genomic DNA (24). The final PCR products were run along with a 1 kb ladder on 1% agarose gel, and their qualities were examined via a gel documentation system (Iran, Padideh Nugen Pars Company, model HU-110). The PCR amplicons were send to Cardiovascular Research Center (Shariati Hospital, Tehran, Iran), and sequenced using BigDye terminator cycle sequencing kit version 3.1 (Applied Biosystems, USA) using manufacturer's instructions. The raw sequences were processed and assembled in Chromas software version 2.1.3 (http://www.technelysium.com.au/ChromasPro.html), accessed on September 18, 2023) to prepare the contigs. The bacterial sequences were registered in the GenBank database get the accession numbers as well as NCBI closely related species to through BLASTn analysis to generate phylogenetic trees. They were reconstructed in MEGAX software using Maximum Likelihood technique and Tamura-Nei model maintaining 500 bootstrap replicates (25). Next, the trees were annotated in Interactive Tree of Life V5 (iTOL, https://itol.embl.de/) (26).

Statistical analysis. The data were reported as Means ± standard error of three or more independent replicates. The analyses of variance (ANOVA) of the cellulolytic abilities were performed in SAS software version 9.1 (27). The significant difference between strain means compared to the negative controls were analysed by Tukey's test; considering $p< 0.05$ as statistically significant.

RESULTS

Screening of cellulose-degrading bacteria. A collection of 64 isolates were obtained from the rumen of the animals (Fig. 1). Up on screening for the CBs, four isolates depicted higher cellulolytic potentials in terms of the clear zone diameter. The higher potential showing isolates viz, Dez wildlife 13A, Dez wildlife 55, Loot desert 111A, and Tabbas desert 32A were selected and coded according to the animals. The isolates like Dez wildlife and13A Loot desert 111A were facultative anaerobes, Gram-negative and rod-shaped bacteria (Fig. 2). In contrast, the strains Dez wildlife 55, and Tabbas desert 32A were found as aerobic, Gram-positive and rod-shaped bacteria (Table 1). For cellulose-degrading potential, the largest and smallest diameter of the clear zone were observed for isolates Dez wildlife 55 (42 mm) and Dez wildlife 13A (12 mm), respectively ($p<0.05$). The maximum HC of cellulose was observed for Dez wildlife 55 (14 mm), whereas the minimum HC was shown by Dez wildlife 13A (6.03 mm).

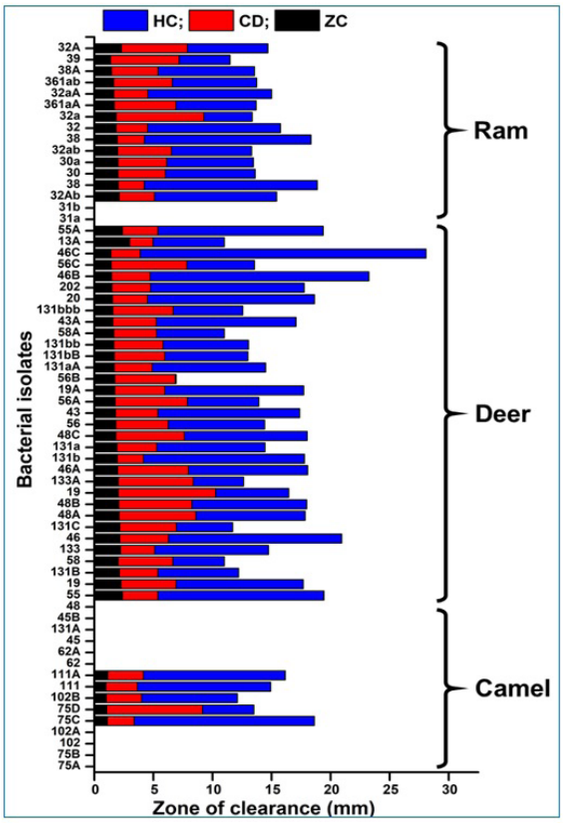

Fig. 1. The cellulose decompose potential and hydrolytic capacity of the isolates. The isolates without bars indicates no activity. HC: hydrolytic capacity; CD: colony diameter; ZC: zone of clearance in millimeters.

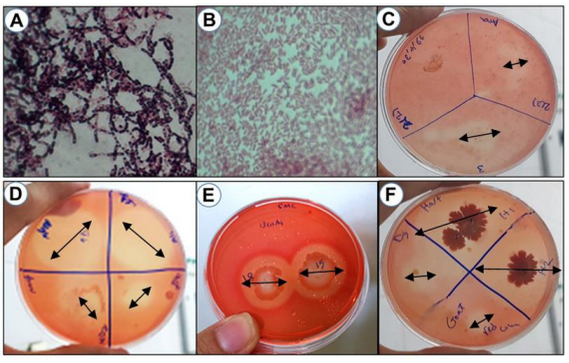

Fig. 2. Gram staining of *B. subtilis* Dez wildlife 55 and C. murliniae Dez wildlife 13A are shown in Figs. A & B. The clear zones around the non-cellulolytic (CA-) and cellulolytic colonies (CA+) on the CMC-NA plates can be seen in figures of C, D, E and F.

As shown in Table 2, the highest CAS was recorded for Dez wildlife 13A (2.98 UmL^{-1}), and the lowest activity was seen for Loot desert 111A (1.14 UmL^{-1}).

Optimization of culture conditions. The optimum temperature for the growth of the cellulolytic isolates was found to be 39°C. Moreover, the strains exhibited resistance to different bilious concentrations (0.3, 1, and 2%) and NaCl (4, 6, and 10%). The Gram-positive isolate, *B. subtilis* Dez wildlife 55 was found acidophilic in nature. In comparison, the *Ornithinibacillus bavariensis* Tabbas desert 32A was recognized as an alkaliphile, which showed higher ability to withstand, and grow under saline conditions. The strain, *Citrobacter murliniae* Dez wildlife 13A grew poorly at the tested pH (Table 1).

Phylogenetic analysis. The molecular homology revealed that the strains belonged to main phyla namely *Firmicutes* and *γ-Proteobacteria* (Fig. 3). Being Gram-negative, the strains like Dez wildlife 13A and Loot desert 111A belonged to the family, *Enterobacteriaceae*. The BLASTn analysis of these bacteria showed over 99% sequence similarity with *C. murliniae* MK9 and *C. braakii* 167, respectively. However, isolates Tabbas desert 32A and Dez wildlife 55 were affiliated with the *Bacillaceae*. The analysis of the assembled sequences indicated that the isolates, Tabbas desert 32A and Dez wildlife 55 were similar to *O. bavariensis* (96.89%) and *B. subtilis* (95.62%), respectively. Based on the BLASTn analysis and phylogenetic reconstruction, the isolates were finally identified and labeled as *C. murliniae* Dez wildlife13A, *C.*

Table 1. The biochemical reactions of cellulolytic bacteria isolated from rumen of camels, deer and rams.

Observations	Bacterial isolates			
	Loot desert 111A	**Dez wildlife 13A**	**Tabbas desert 32A**	**Dez wildlife 55**
Isolation source	CR	DR	RR	DR
Sampling method	AS	ST	AH	ST
Growth condition	A	A	A	A
Pigment production	gray	gray	slightly brownish	cream
Shape	rode	rode	rode	rode
Gram staining	-	-	+	+
Spore-forming	-	-	Central	Central
Snot test	+	+	-	-
Oxidase reaction	-	-	-	-
Catalase test	+	-	+	+
Indole production	-	+	-	-
Gas production	-	+	-	-
H2S production	+	+	-	-
Motility test	+	+	-	+
Citrate consumption	-	+	-	-
Lactose fermentation	-	-	-	-
Growth on EMB	+	+	-	-
Esculin hydrolysis	-	-	+	+
Starch hydrolysis	-	-	-	+
Temperature optimization (°C)				
27	+++	++++	++++	+++
33	++++	+++	++++	++++
39	++++	++++	++++	++++
45	++++	++++	++++	++++
pH optimization				
2.5	++	++	+++	+++
6.8	++	++	++	++
7.4	+++	++	++++	++
8	++	++	+++	++
NaCl tolerance (W/V)				
4%	+++	++	+++	+++
6%	+++	++	++++	+++
10%	+++	++	+++	++
Bile tolerance (W/V)				
0.3	+++	+++	+++	++
1	+++	++	+++	+++
2	++	+++	+++	++
Primary identification	*Citrobacter*	*Citrobacter*	*Ornithinibacillus*	*Bacillus*
Accession number	ON323018	ON323019	ON323020	ON323021

CR, Camel rumen; DR, Deer rumen; RR, Ram rumen; AS, After slaughtering; ST, Stomach tube; AH, After hunting; FA, Facultative anaerobic; A, aerobic; +: positive, -: negative; ++++: potent, +++: moderate, ++, weak, +: very weak; Eosin methylene blue (EMB)

braakii Loot desert 111A, *O. bavariensis* Tabbas desert 32A and *B. subtilis* Dez wildlife 55, respectively.

Enzymatic activities of the isolates. The highest and lowest potential to hydrolyze tannin was recorded by *O. bavariensis* Tabbas desert 32A (30.23 mm) and *B. subtilis* Dez wildlife 55 (14.66 mm), respectively ($p<0.05$). Compared to other isolates (0.00), *B. subtilis* Dez wildlife 55 showed a potent potential for AA activity (30.00 mm) ($p<0.05$) as shown in the (Table 2). Among them, *C. murliniae* Dez wildlife 13A (34.53 mm) had the highest ability to degrade protein, while the lowest potential was observed for *B. subtilis* Dez wildlife 55 (16.13 mm) ($p<0.05$). All the tested enzy-

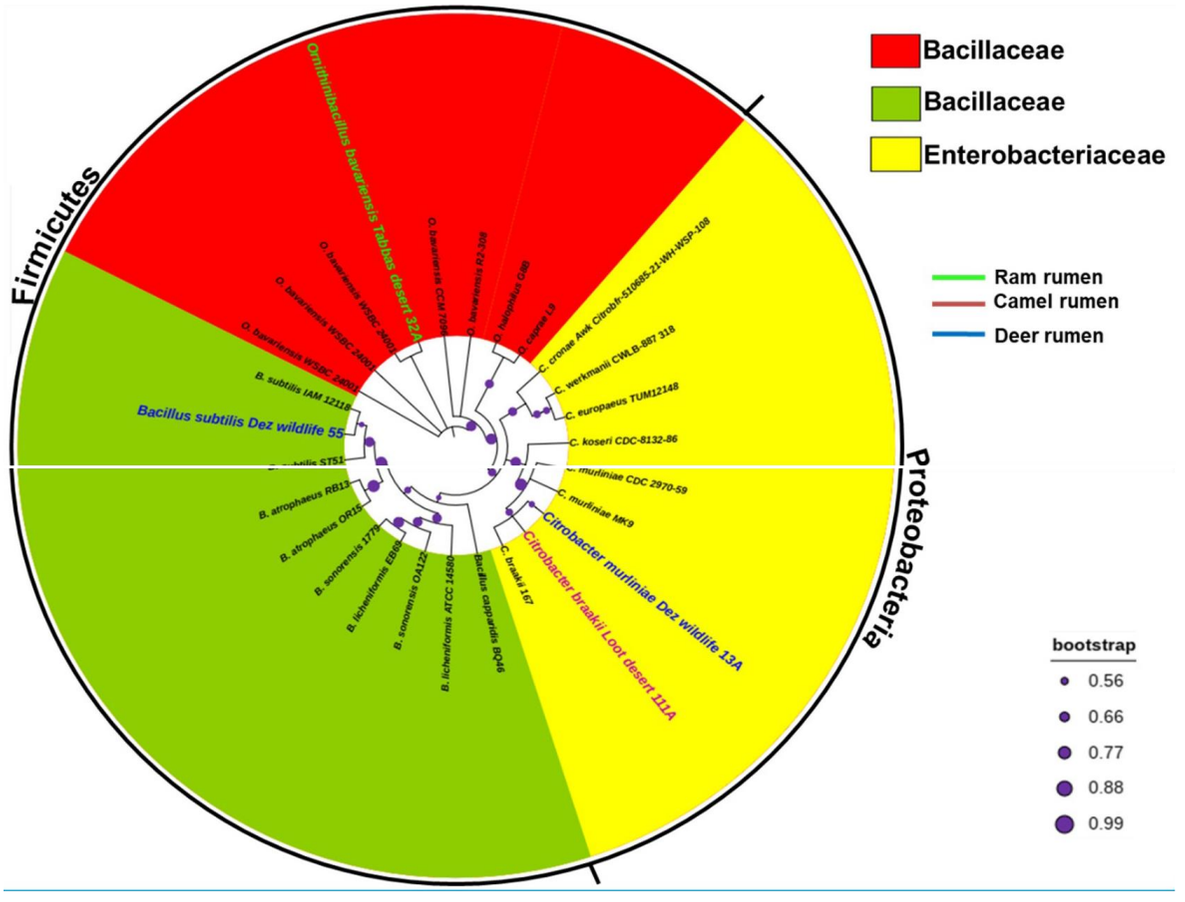

Fig. 3. Phylogenetic reconstruction of the bacteria isolated from the rumen of URs, BCs and FDs. The symbols at nodes represent fraction of the bootstrap values from 500 replications with values only above 50%.

Table 2. The hydrolytic potential of isolates identified in rumen of ram, deer and camel.

Isolate	CAS** (U/mL^{-1})	Hydrolysis capacity (HC)	Zone of clearance (mm)			
			CAS	TA	AA	PA
C. braakii Loot desert 111A	1.14^{c}	12.00^{a}	36.10^{b}	19.20^{c}	0.00^{b}	28.10^{b}
C. murliniae Dez wildlife 13A	2.98^{a}	6.03^{b}	12.00^{c}	24.10^{b}	0.00^{b}	34.53^{a}
O. bavariensis Tabbas desert 32A	2.28^{b}	6.81^{b}	38.10^{ab}	30.23^{a}	0.00^{b}	26.23^{b}
B. subtilis Dez wildlife 55	2.38^{b}	14.00^{a}	42.00^{a}	14.66^{d}	30.00^{a}	16.13^{c}
SEM*	0.11	0.45	0.87	0.88	0.10	0.43
p-value	0.001	0.001	0.001	0.001	0.001	0.001

*SEM, standard error of means; ** Different superscripted lowercase letters indicate the statistically significant differences among the enzyme activities of the isolated bacteria at the $p<0.05$ level. In a column, the enzyme strength of the isolates decreases in alphabetical order (from a to z).

matic abilities of the isolated strains are demonstrated in the (Table 1 and Fig. 4).

Antibacterial and antibiogram activities. As shown in Table 3, *C. braakii* Loot desert 111A (14.00 mm) had a high inhibitory effect against *S. aureus*, while *C. murliniae* Dez wildlife 13A (0.00 mm) showed the lowest effect ($p<0.05$). Besides, the growth of the pathogenic *E. coli* was strongly suppressed by *C. murliniae* Dez wildlife 13A (12.33 mm), while *O. bavariensis* Tabbas desert 32A did not affect it ($p<0.05$). Against *P. aeruginosa*, the best inhibition activity was recorded by *C. braakii* Loot desert 111A (12.06 mm). However, *O. bavariensis* Tabbas desert 32A did not show any action against *P. aeruginosa*. For inhibiting pathogen *B. subtilis*, *C. murliniae* Dez wildlife 13A (59.20 mm) and *B. subtilis* Dez wildlife 55 (8.30 mm) showed the strongest and weakest inhibition. The results of antibiotic susceptibility test are presented in Table 3. All the bacteria, except *O. bavariensis* Tabbas desert 32A (10.00 mm), demonstrated antibiogram activities (AS) against furazolidone ($p<0.05$). In addition, the most sensitive and resistant isolates to kanamycin were *O. bavariensis* Tabbas desert 32A (30.06 mm) and *B. subtilis* Dez wildlife 55 (12.00 mm), respectively. The highest antibiotic susceptibility against amoxicillin, was observed with *C. murliniae* Dez wildlife 13A (24.26 mm), while *O. bavariensis* Tabbas desert 32A (12.20 mm) showed the lowest susceptibility to it ($p<0.05$). Among the isolates only *O. bavariensis* Tabbas desert 32A showed sensitivity to rifampin depicting a zone of inhibition as big as 25.3 mm in diameter.

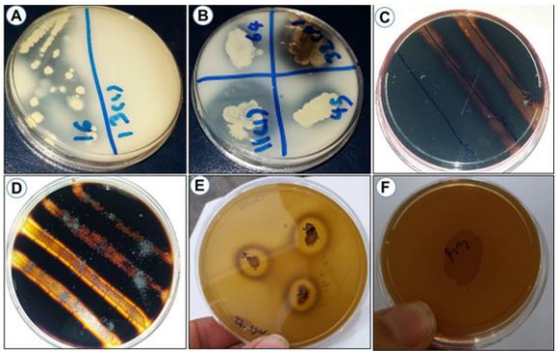

Fig. 4. The enzymatic potential of the isolates with bright halos around the colonies. A & B, proteolytic activity; C & D, amylase activity; E & F, tannase activity. Colonies without halo are negative controls for each of the enzyme activities.

DISCUSSION

Bioprospection of the microbes can lead to the discovery of promising and novel bacteria with biotechnological uses. Recently, significant progress has been made in new and safe food additives production technologies. In addition to being compatible with the environment, sustainability and reducing the economic costs of feed, they should improve the health and performance of domestic animals. During the last's century, the ruminants like cow and sheep have proved as significant resources for biocatalysts

Table 3. The antibacterial activity and antibiotic response of the cellulolytic strains.

Isolate*	Inhibition zone** (mm)							
	Pathogenic strains				Antibiotics			
	S. aureus[1]	*E. coli*[2]	*P. aeruginosa*[3]	*B. subtilis*[4]	FR[5] 100µg	KAN[6] 30µg	AMX[7] 25µg	[8]RA 5µg
C. braakii Loot desert 111A	14.00^{a}	8.16^{c}	12.06^{a}	18.06^{b}	0.00^{b}	20.13^{b}	22.00^{b}	0.00^{b}
C. murliniae Dez wildlife 13A	0.00^{c}	12.33^{a}	8.50^{b}	59.20^{a}	0.00^{b}	22.00^{b}	24.26^{a}	0.00^{b}
O. bavariensis Tabbas desert 32A	8.00^{b}	0.00^{d}	0.00^{d}	9.5^{c}	10.00^{a}	30.06^{a}	12.20^{c}	25.33^{a}
B. subtilis Dez wildlife 55	8.00^{b}	10.00^{b}	6.00^{c}	8.3^{c}	0.00^{b}	12.00^{c}	12.30^{c}	0.00^{b}
SEM*	0.14	0.18	0.32	0.43	0.00	0.66	0.39	0.16
p-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

[1]*S. aureus*; *Staphylococcus aureus*; [2]*E. coli*, *Escherichia coli*; [3]*P. aeruginosa*, *Pseudomonas aeruginosa*; [4]*B. subtilis: Bacillus subtilis*; [5]FR, furazolidone 100µg; [6]KAN, kanamycin 30µg; [7]AMX, amoxicillin 25µg; [8]RA, rifampin 5µg. *SEM, standard error of means; ** Uppercase lowercase letters indicate a significant difference between the activities of the isolates at the level of $p<0.05$.

with probiotic applications (10, 11). In this context, the physiologically diverse rumen of animals like FDs, URs and BCs can serve as the unique resources for bioprospection of novel bacteria having industrial potentials (3-9).

The current research highlights the identification of probiotic bacteria from the unexplored animals like URs, BCs and FDs using molecular sequencing-based methods. The homology based on 16S rRNA gene sequencing affiliated the isolated CBs to the genera of *Bacillus*, *Ornthinibacillus*, and *Citrobacter*. According to the findings, these CBs included Gram-positive and Gram-negative rods. Our observations are supported by previous studies (3-9, 12, 28-30) that reported the isolation of Gram-positive *bacilli* and *coccobacilli* with cellulose degradation capabilities. To date several CBs such as *B. subtilis*, *B. pumilus*, *Achromobacter*, *Paenibacillus* sp. etc have been isolated from the RCs of goat, sheep, buffalo, cattle and panda (3-9, 31-33). Moreover, the present study selected the RCs of BCs, FDs and URs, which can serve as the treasure-trove of efficient bacterial species with probiotic potentials to digest fibrous polymers. In the current study, isolates Dez wildlife 55 and Dez wildlife 13A showed the potent HC and CA, respectively. Other study was reported the halo diameter of the cellulolytic activity between 2 and 6 mm (34). The halo diameter of cellulose decomposition by isolate Dez wildlife 55 was seen in the range of 41-47 mm, and the HC was 1.81- 2.67 mm. Rawway et al. (35) also introduced two cellulolytic strains of *B. subtilis* with CAS activities in ranging 9.637 and 12.152 UmL^{-1}. The CAS activity of *Bacillus* isolates was equal to 0.02, 0.04, 0.07, 0.53, 1.78 and 3.09 UmL^{-1} (36, 37). The difference in the reported enzymatic activities may be caused due to variation of substrates, incubation time, temperature, pH, bacteria source, or culture conditions (34-38). Moreover, the highest cellulase activities were detected with isolate *B. subtilis* Dez wildlife 55 at a pH 7 and temperature of 39°C that corresponded to the physicochemical conditions of the rumen systems. Similar results have been reported for maximum CAS activities at pH of 7 by Potprommanee and coworkers (39).

To minimize or avoid the use of toxic chemicals (40), the use of animal probiotics has gained scientific attention recently to maintain health of animals (10). This experiment was isolated four CBs from the rumen of BCs, FDs and URs that exhibited probiotic potentials.

The biochemical characterization of the CBs demonstrated significant activities at a broad range of pH that corresponds to the intestinal environments of the ruminants. The isolates *O. bavariensis* Tabbas desert 32A and *B. subtilis* Dez wildlife 55 showed more favorable growth in bilious and acidic environments. Similar bacteria such as *B. licheniformis*, *Enterococcus hirae*, and *Pediococcus acidilactici* have been isolated from the same source and show resistance to bile and acidic conditions (41). The tolerance and growth of *O. bavariensis* Tabbas desert 32A at varied pH could be attributed to its sporulation capacity. Moreover, the acid tolerance of these isolates could be due to their origin and H+-ATPase activity (9). Further, the tolerance to stressful and acidic conditions signposts them as suitable candidates for probiotic applications (42). For probiotics the autochthonous bacteria isolated from the same host are preferred for being more compatible with the intestinal microbiota and prevailing micro-environmental conditions (9). Naeem et al. (43) observed that probiotic *Bacillus* was compatible with acidic (1 to 4) and bile (0.3%) conditions, as previously (44). All the bacteria except the ones isolated from the deer rumen were halophilic depicting tolerance to high salt concentrations of over 3.5%. The halophilic *O. bavariensis* strains tolerant to high salt concentrations were previously isolated from soils and hypersaline lakes (45-47).

Among the probiotic candidates, *Bacillus* has ability to sporulate, which enable them to survive in conditions like gut. Besides, it can suppress pathogens by producing antimicrobial materials, and is non-toxic for animal feeding, which making them more suitable probiotic compared to other microorganisms (10). Some of the enzymes including proteases, galactosidases, xylanases, phytases, and amylases are very important in raising poultry and pigs (48). The reasonable microorganism for producing feed enzyme must be non-pathogenic and non-toxigenic like ones reported in the study. In some terrestrial and aquatic animals, *Bacillus* enzymes has absorbed nutrients from poorly digested plant resources (10). The diverse enzyme activities of probiotic agents hold much attention since they have a significant effect on feed digestion. The *C. murliniae* Dez wildlife 13A was the best proteolytic strain, while the starch was degraded by *B. subtilis* Dez wildlife 55 only. However, the highest TA was shown by *O. bavariensis*

Tabbas desert 32A. Our results are supported by previous studies (18), who reported tannase, protease, amylase, xylanase, lipase, and endoglucanase activities for the bacteria extracted from the koala's intestine. The members of *Ornithinibacillus* and *B. subtilis* isolated from the ruminants are well known to show tannase, protease, amylase and lipase activities (49-51). Recently, a proteolytic strain, *O. caprae* has been tested to degrade poultry feathers (52). Naeem et al. (43) stated that *Bacillus* strains effectively suppressed pathogens. In the current study, the isolates had a favorable AA against pathogens, as a paramount ability for probiotics, which can be due to the production of antimicrobial matter including organic acids, H_2O_2, and bacteriocin (53). The isolated bacteria showed considerable sensitivity to different antibiotics being highest by *O. bavariensis* Tabbas desert 32A to rifampin. Similar results have been stated for gut microbes isolated from other animals. Further, the gut bacteria like *Lactobacillus, Bacillus* and *Enterococcus* species are known to show antibiotic resistance (10). The noteworthy finding of the study is the discover of *O. bavariensis* in the digestive system of an animal, URs, for the first time, which has cellulolytic, antimicrobial and proteolytic activities. Previously, *Ornithinibacillus* strains were isolated from the other sources such as Bavarian pasteurized milks, littoral sediments (54), person's blood samples (55), dead ship oysters (56), super salty lake water (47), slush compost (57), alkaline soils with high salinity (46), loch sediment (45), and goat hide (52). The current research used combined morphological, biochemical and molecular techniques for a deeper understanding of cellulose degradation in the rumen microbiome and was able to introduce potential probiotic cellulolytic bacteria. However, animal studies seem necessary for their practical application.

CONCLUSION

The present study highlights the ruminal CBs with a good potential to digest starch, tannin, and protein besides being highly active for cellulose bioconversion. The CBs were resistant to acidic and bilious conditions, and showed excellent AA. More in-depth research is needed to claim that *O. bavariensis* is an excellent metabolic partner for URs that increases their capacity to digest CBM. Further studies could help to clarify whether the isolates are indeed effective in degrading cellulosic biomass by cloning the genes encoding individual enzymes. Nonetheless, the overall findings suggest *C. murliniae* Dez wildlife 13A as a specialized cellulase producing biocatalyst, while *B. subtilis* Dez wildlife 55 as different enzyme producer. Moreover, the cellulolytic bacterium, *O. bavariensis* Tabbas desert 32A with potential probiotic activities was isolated for the first time from the rumen of Urial rams.

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