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(Research Paper)

Tannase-producing Bacteria Isolated from the Rumen of Fallow Deer (*Dama dama*): Livestock Potential Feed Additives

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Abstract

Introduction: Tannins are a group of polyphenolic compounds that are widely present in plants as an anti-nutritional factor. The rumen of wild ruminants contains novel microbes that detoxify antinutrients and improve feed digestion. The present study evaluated tannase-producing bacteria isolated from the rumen of Fallow deer (*Dama dama*), livestock potential feed additives.

Materials and Methods: Tannase-producer bacteria (TPBs) were isolated from the rumen using a 2% tannic acid- plate and tannase activity (TAA) assayed by the spectrophotometer method. The bacterial DNA was extracted through boiling and amplified using a PCR reaction. The Sanger technique and BLAST software were used to identify the strains.

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Antibacterial (ABA) and antibiogram tests were performed by the disc diffusion method, and the acid and bile resistance of isolates were examined using broth cultures.

Results: The results indicated that TPBs belonged to *Klebsiella*, *Enterobacter*, and *Escherichia* genera. *Escherichia fergusonii* GHMGHE44 (9.39 Uml^{-1}) and *Enterobacter cloacae* GHMGHE26 (1.79 Uml^{-1}) were the strongest and weakest tannin degraders ($p<0.01$). Among the isolates, bile and acid resistance were insignificant ($p>0.01$) but *E. fergusonii* GHMGHE28 (9.48 CFU ml^{-1}) had a significant survival rate compared to *E. cloacae* GHMGHE25 (9.07 CFU ml^{-1}) at pH of 7 ($p<0.01$). Also, *K. pneumoniae* subsp. *rhinoscleromatis* GHMGHE27 (32.66 mm), *E. coli* GHMGHE47 (40.66 mm), and *E. fergusonii* GHMGHE48 (24.66 mm) were potently suppressed the pathogen *E. Coli*, *S. aureus* and *P. aeruginosa*, respectively ($p<0.01$). Against used antibiotics, *E. asburiae* GHMGHE22 was the most sensitive isolate while others showed diverse reactions ($p<0.01$).

Discussion and Conclusion: The findings showed that TPBs have the potential to study as commercial animal feed additives (AFA).

Keywords: *Escherichia fergusonii*, Tannase, Cellulase, European Fallow Deer

Introduction

Over the past two decades, many studies have been performed to detect the relationship between animals and their gut microbiomes. New findings have shown that diet, phylogeny, and genetics of the host are the most important factors affecting the composition and function of the host's gut microbiome (1). A question: Is the intestinal microbiota of animals influenced by their habitat? Some studies found that about 20% of the amphibian gut microbiome may have originated from water sources in spring, while only about 5% came from water sources in autumn (2). In the natural world, many animal species exist and the stability of their gut microbiome is influenced by their diet. It is one of the most important factors affecting the stability of gut microbiome composition and function. For example, the gut microbiome of the bamboo-eating giant panda (*Ailuropoda melanoleuca*), a pure bamboo diet contains hardly any protein and a lot of indigestible fiber, can easily digest cellulose and detoxify cyanide compounds (3). Here, browsing animals encounter woody plants that have lower cell walls and relatively higher lignin and plant secondary metabolites. Consequently, browsers consume primarily woody plants up to 70% that contain higher protein than grasses. But, this protein is often unavailable for digestive reactions due to binding with plant secondary metabolites (PSMs). Tannins, a type of PSM, is known to be an anti-nutritional factor that has diverse deleterious effects when ingested by animals, although these disadvantages depend on the plant species and tannins-consuming animal (4). The most harmful effect of tannins on animals is a reduction in protein availability and dry matter digestibility. As a strategy to reduce the impact of tannins, browsing animals produce the salivary proteins, proline, that bind to tannins of forage during mastication in the oral cavity (4). Another strategy is thatsome gut microbes - break down and neutralize tannin by secreting tannase enzyme (5). This ability has been found abundantly in the rumen of tannin-browser species including mule deer (*Odocoileus hemionus*), moose (*Alces alces*), Duiker (*disambiguation*), steenbok (*Raphicerus campestris*), klipspringer (*Oreotragus oreotragus*), Urial ram (*Ovis vignei*), and fallow deer (*Dama dama*). However, grazer animals such as sheep (*Ovis aries*) and cows (*Bos taurus*) do not have this potential (4, 6-8). Many studies

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suggested that microbes contain enzyme-coding genes and enzymes can be used to combat the lack of animal feed, as microbial additives (9-14). In tropical and subtropical countries, dietary requirements of livestock are fulfilled through some fibrous forage and agricultural waste that contain high-molecular weight tannin. These compounds lead to poisoning and even death of the animal if added to the diet at a level higher than 5% (15). Hence, tannin-degrading bacteria may detoxify tannins by producing tannase, tannin acyl hydrolases (E.C.3.1.1.20). It is an inducible enzyme that catalyzes the hydrolysis of ester and depside linkages of gallotannins and other tannins, and results in producing gallic acid and glucose (16). TPBs have extensive uses in the food, beverage, brewing, pharmaceuticals, chemicals, fruit juices, and animal feed industries to produce gallic acid, pyrogallol, propyl gallate, methyl gallate, and tea, in clarification of beer and manufacture (16). Also, it can be an analytical probe, to determine the structures of naturally gallic acid esters, and in the treatment of tannin-containing effluents in olive and tannery factories (16). To solve an old challenge, this study focused on the rumen microbiome of the European fallow deer that consumed high-tannin forages and could tolerate tannin with no problem. The current study evaluated TPBs isolated from the rumen of Fallow deer (*Dama dama*): livestock potential feed additives.

Materials and methods

Habitant of Deer: Dez Kupitte is a large natural history museum and wildlife park in Iran (Khuzestan province, Dezful), located in the vicinity of the roaring Dez River with an area of 6 hectares. The highest and lowest annual temperature varies between zero and 60 °C. We selected four heads of deer for sampling (2 males and 2 females). They were browsed on the pasture plants, including *Conocarpus erectus*, *Alhagi persarum*, *Ficus carica*, *Acacia nummulorii*, *Astragalus* sp., *Albizia lebbeck*, *Olea europaea*, and *Eucalyptus microtheca* leaves, and also fed by manual diets containing alfalfa, wheat bran, and barley twice a day for 6 months. Animals had free access to water. All animal management and sampling procedures were conducted according to the care and use of agricultural animals in research and teaching guidelines (17).

Isolation of Tannase-Producer Bacteria: This study was conducted at the University of Birjand. The rumen fluids of deer were collected using a stomach tube and a vacuum pump. The various dilutions (10^{-1} to 10^{-10}) of rumen fluid were cultured on nutrient agar (NA) media and incubated at 39°C for 2 d under aerobic and anaerobic conditions (18, 19). After sequent sub-cultures on NA, colonies with different forms were selected. The bacterial colonies were streaked on NA and stored at 4°C. The pure colonies were spotted onto the 2% Tannic acid-NA medium and incubated at 39°C for 2 d (18-19). Next, each plate was stained with 15 ml of $FeCl_3$ (0.1 M) for 30 min, washed, and decolorized with pure water (2–3 times). In the end, the clear zone diameter was measured by a ruler (mm), and strains with the best hydrolysis activity of tannic acid were chosen for further analysis (18-19).

Tannase Assay: The screened isolates were inoculated in 0.2% gallic acid-nutrient broth (NB) medium for 2 d at 39°C with shaking (150 rpm min^{-1}), and TAA was quantified. One mL of the cultured isolate is taken and centrifuged at 8000 g min^{-1} for 15 min. One ml of 0.3 mM tannic acid in 0.1 M citrate buffer (pH 3.5) was mixed with 0.1 ml of supernatant and incubated for 30 min at 39°C. The reaction stopped by adding 0.2 ml HCl (2 N). In the control tube, 0.2 ml of 2 N HCl and 0.1 ml of supernatant were mixed with and placed in the incubator at 39°C for 30. Then, 1 ml of 0.3 mM tannic acid in 0.1 M citrate buffer was added to the control tube. In the blank tube, 0.1 ml of 0.1 M citrate

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buffer was mixed with 1 ml of 0.3 mM tannic acid solution (in 0.1 M citrate buffer). After 30 min, 0.2 ml of 2 N HCl was added to the blank. The gallic acid released during hydrolysis of tannic acid represented the extracellular tannase activity. The rhodanine reaction was used to measure gallic acid. Also, 300 µl of rhodanine solution (0.667% in methanol) was added to 200 µl of the standard tube and vortexed. After 5 min, 4.5 ml of aqueous KOH (0.5 M) solution was added to the standard sample and incubated for 20 min. The absorbance of the tubes was read at 620 nm. The unit (U) of TAA is equal to one µmol of gallic acid released per minute (20).

$$\Delta A = \text{Absorbance (Test} - \text{Blank)} - \text{Absorbance (Control} - \text{Blank)}$$

Bacterial Identification: After gram staining, the isolates were studied using a light microscope to observe the colony's morphology i.e., color, shape, and size. The bacterial isolates were characterized by biochemical tests (21), and the boiling method was used to extract their genomic DNA (22). The reaction mixture of PCR prepared using the genomic DNA, forward and reverse primers (27F, 5′AGAGTTTGATCCTGGCTCAG- 3′; 1492R, 5- GGTTACCTTGTTACGACTT-3), master mix 2X (Parstus; Iran), and deionized water. Then, 16S rDNA gene amplification of selected isolates was performed in a Bio-Rad i-cycler (Bio-Rad, Hercules, CA), as explained by Weisburg et al. (23). A partial sequence of the 16S rDNA gene was determined and compared to those in the National Center for Biotechnology Information (NCBI), Gene Bank database, using the BLAST program. The phylogenetic tree of sequences was drawn by MEGAX software (24-26).

Enzyme Potentials: The potential of isolates for producing cellulase, amylase, and protease was evaluated by plate assay (11). For amylolytic potential, NB was supplemented by agar (2%) and starch (1%). The plates were inoculated with pure isolates and incubated (at 39 °C for 48 h). After that, iodine crystals were sprinkled over the Petri dish, and amylase activity was detected by a luminous zone around the colony (11). The cellulolytic activity was studied by adding 2% agar and 1% carboxymethyl cellulose (CMC) in 100 ml of NB medium. After spotting on the center of the plate (a loopful of bacterial suspension), they were incubated at 39 °C for 48 h. At the end, the plates were stained with 0.1% Congo red solution and then washed with physiology serum. The clear halo around the inoculation point was considered as positive cellulase (11). Also, proteolytic activity was evaluated by agar media containing 1% bovine serum albumin. After spot inoculation, the plates were placed in an incubator for 48 h and immersed in the solution of 1% glacial acetic acid. Bright zone formation around the colonies indicated proteolytic potential (11).

Antibiogram and Antipathogenic Tests: Antibiotic resistance (ABR) was examined by the disk diffusion method (26). The strains were cultured in NB media at 39 °C for 1 d. The bacterial suspensions were swabbed on the Mueller Hinton agar (MHA) plates. Using sterile forceps, the antibiotic disks including amoxicillin 20 µg, cefixime 5 µg, ciprofloxacin, and gentamicin 10 µg (6 mm diameter; Padtan Teb, Iran) were placed on the MHA plates and incubated at 39 °C for 2 d. The inhibition zone around the disks was measured (mm), as antibiotic susceptibility (26).

For ABA (11), 100µl of each indicator strain (*E. coli* ATCC 13706, *S. aureus* ATCC 9027, and *P. aeruginosa* ATCC9027) were spread on MHA plates. For each strain, three sterile disks impregnated with the bacterial supernatant were prepared and set on MHA plates (incubated at 39$^{\circ C}$ for 1 d). The presence of a clear zone around the disks was measured (mm), as ABA. A sterile blank disk (6 mm) impregnated with MiliQ water was

considered as a negative control (11).

Bile and Acid Resistance Test: The bile resistance was evaluated using NA plates supplemented with bovine-sheep bile at concentrations of 0.5, 1, and 2% (w/vol), a loopful of each isolate was spotted onto NA-bovine-sheep bile plates and incubated aerobically for 48 h at 39 °C, as described by Maldonado et al. (10). The isolates were cultured into tubes containing NB (at 39 °C for 1 d) and their growth was compared with the control tube (without bile), (10). For acid resistance (20), the pH of sterile phosphate-buffered saline (PBS) was adjusted to 2.5 and 7 using HCl and NaOH. Then, each isolate was inoculated separately in tubes with acidic and neutral pH and incubated at 39°C for 1 d. The bacterial cultures were serially diluted in the sterile PBS solution (10-fold) and the survival rate (SR) of isolates was calculated as log values of colony-forming units per ml (CFU ml^{-1}) (20).

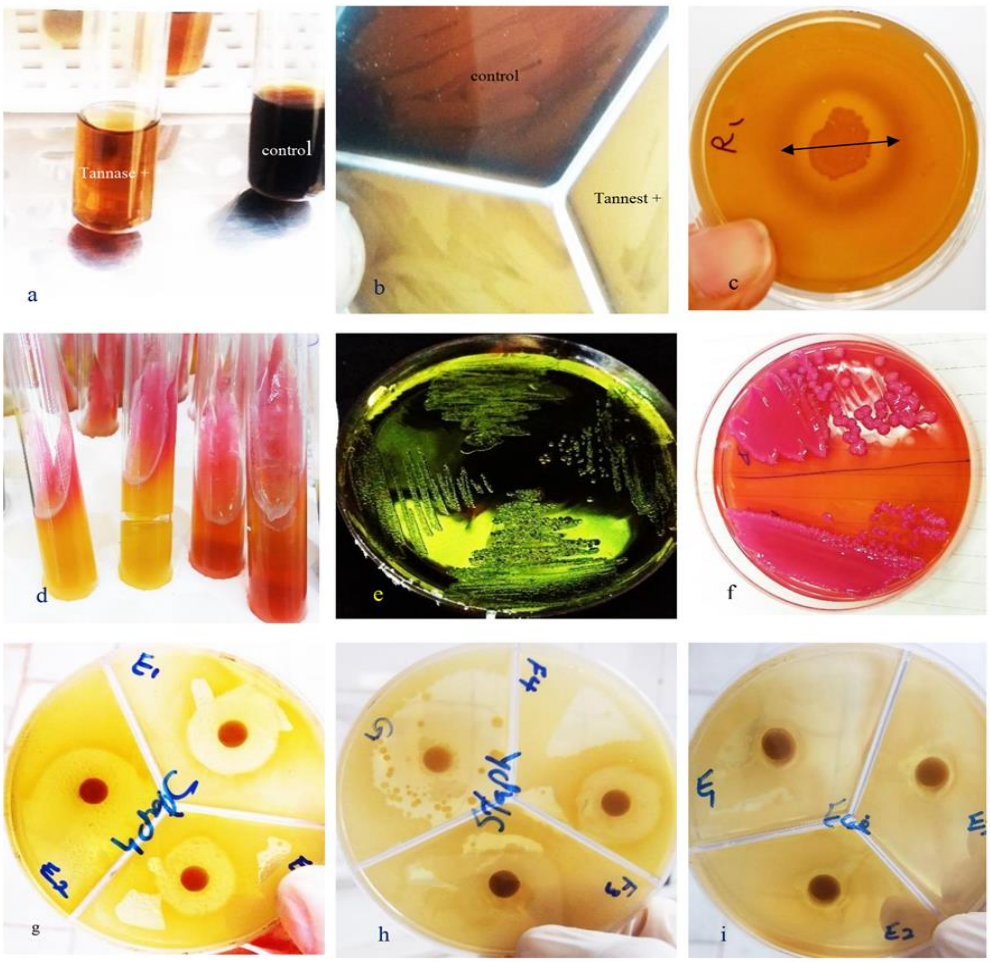

Fig. 1- Tannase assay using broth (a) and agar media (b), and a clear halo around the colony as tannase potential (c); Biochemical reactions of the isolates: Triple sugar iron agar test (d), Eosin methylene blue culture (e), and growth on MacConkey agar (f); Antibacterial effect of the isolates against *Staphylococcus aureus* (g), *Staphylococcus aureus* (h), and *E. coli* (i)

Statistical Analysis: The results of the biochemical test were evaluated using tables of Rosenberg et al. (21). The results of antibiotic suceptibility were interpreted using the Clinical and Laboratory Standards Institute (CLSI) tables. Nucleotide sequences were edited using the software of Chimera 1.15 and analyzed by the BLAST program. The MEGAX software was used for drawing the phylogenetic tree (24-25, 27-28). All quantitative parameters were analyzed by

SAS (29) software (ver. 9.4), proc GLM, to statistically detect differences among isolates using the Tukey-Kramer test with a significance level of 0.05.

Results

Tannase Producer Bacteria: On 2% tannic acid-NA plates, the presence of a clear zone around colonies was considered as the production of tannase (Fig. 1a, 1b, 1c). This study isolated fifteen tannase-producer rods from the rumen of deer that were gram-negative lactose-fermenting bacteria (Table 1). Their activities on the TSI agar plates were Acid/Acid (Fig. 1d, 1e, 1f). The strains of *Escherichia* were indole-producer. Among them, GHMGHE44 had the maximum ability to hydrolyze the tannic acid (9.39 U ml^{-1}) but GHMGHE26 (1.78 U ml^{-1}) recorded the minimum activity ($p<0.05$).

Table 1- Biochemical Reactions, Tannase Activity, and Molecular Identification of Bacteria Isolated from the Deer Rumen

Isolate	GS	IN	LA	CA	SN	TSI	OX	MR	VP	TAA*	Identification	GenBank accession
GHMGHE21	-	+	+	+	+	A/A	-	-	+	6.02^{d}	*Klebsiella michiganensis*	MZ891607.1
GHMGHE22	-	-	+	+	+	A/A	-	+	-	3.59^{f}	*Enterobacter asburiae*	MZ891608.1
GHMGHE23	-	-	+	+	+	A/A	-	-	-	7.32^{c}	*Enterobacter cloacae subsp dissolvens*	MZ891609.1
GHMGHE24	-	-	+	+	+	A/A	-	-	-	8.27^{b}	*Klebsiella pneumoniae*	MZ891610.1
GHMGHE25	-	-	+	+	+	A/A	-	-	-	1.96^{h}	*Enterobacter cloacae*	MZ891611.1
GHMGHE26	-	-	+	+	+	A/A	-	-	-	1.78^{h}	*Enterobacter cloacae*	MZ891612.1
GHMGHE27	-	-	+	+	+	A/A	-	-	-	3.50^{fg}	*Klebsiella pneumoniae subsp. rhinoscleromatis*	MZ891613.1
GHMGHE28	-	+	+	+	+	A/A	-	-	-	4.13^{f}	*Escherichia fergusonii*	MZ891614.1
GHMGHE43	-	+	+	+	+	A/A	-	+	-	8.75^{a}	*Escherichia fergusonii*	MZ891629.1
GHMGHE44	-	+	+	+	+	A/A	-	+	-	9.39^{ab}	*Escherichia fergusonii*	MZ891630.1
GHMGHE45	-	+	+	+	+	A/A	-	+	-	2.84^{g}	*Escherichia fergusonii*	MZ891631.1
GHMGHE46	-	+	+	+	+	A/A	-	+	-	3.65^{f}	*Escherichia fergusonii*	MZ891632.1
GHMGHE47	-	+	+	+	+	A/A	-	+	-	6.61^{cd}	*Escherichia coli*	MZ891633.1
GHMGHE48	-	+	+	+	+	A/A	-	+	-	5.20^{e}	*Escherichia fergusonii*	MZ891634.1
GHMGHE49	-	+	+	+	+	A/A	-	+	-	2.04^{h}	*Escherichia coli*	MZ891635.1
SEM										0.14		
p-value										0.0001		

GS: Gram stain; IN: Indole; LA: Lactose; CA: Catalase; SN: Snot; TSI: Triple sugar iron agar; OX: Oxidase; MR: Methyl Red; VP: Voges-Proskauer; TAA: Tannase activity; *All means with different Latin alphabets has statistically categorized in different groups ($p<0.01$).

Molecular Identification: Based on the 16S rDNA analysis, isolates GHMGHE21, GHMGHE22, GHMGHE23, GHMGHE24, GHMGHE25, GHMGHE26, GHMGHE27, GHMGHE28, GHMGHE43, GHMGHE44, GHMGHE45, GHMGHE46, GHMGHE47, GHMGHE48, and GHMGHE49 had the highest similarities to *Klebsiella. michiganensis* (96.49%), *Enterobacter asburiae* (97.79%), *Enterobacter cloacae subsp. dissolvens* (97.04%), *Klebsiella pneumoniae* (97.04%), *Enterobacter cloacae* (98.15%), *Enterobacter cloacae* (98.15%), *Klebsiella pneumoniae subsp. Rhinoscleromatis* (97.04%), *Escherichia fergusonii* (100%), *Escherichia fergusonii* (100%), *Escherichia fergusonii* (100%), *Escherichia fergusonii* (100%), *Escherichia fergusonii* (100%), *Escherichia coli* (99.58), *Escherichia fergusonii* (100%), and *Escherichia coli* (99.63%), respectively. The phylogenetic tree of isolates was constructed using 15 representative strains by a Neighbor-Joining method (Fig. 2).

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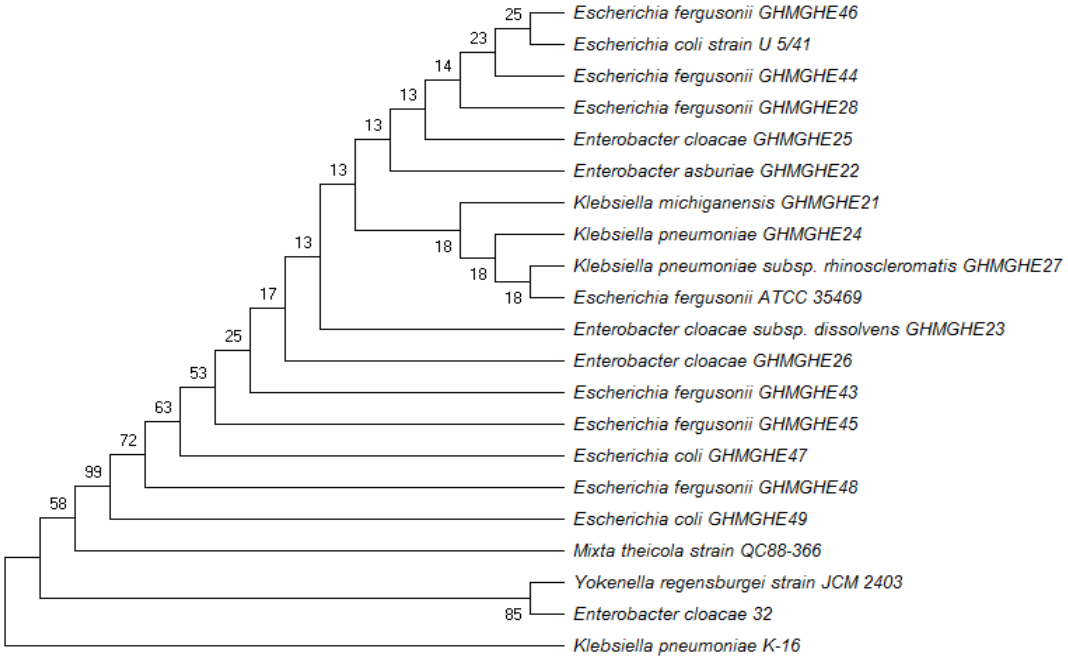

Fig. 2- The evolutionary history was inferred using the Neighbor-Joining method (27) in the bootstrap test (500 replicates) are shown next to the branches (28). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (25) and are in the units of the number of base substitutions per site. This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 299 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (24).

Hydrolytic Activity, Bile, and Acidic Resistance: The cellulase, protease, and amylase potentials varied among the isolates, and three enzyme potentials were seen only for *E. fergusonii* GHMGHE44 and *E. coli* GHMGHE47 (Table 2). On NA media containing 0.5, 1, and 2% bile salts, TPBs were grown easily. There was an insignificant difference between isolates for growth in acidic environments ($p>0.05$). Compared to *E. cloacae* GHMGHE25 (9.07 CFU ml^{-1}), *E. coli* GHMGHE49 had the highest SR at pH of 7 (9.21 CFU ml^{-1}), ($p>0.05$).

Antibiogram Activity: *E. cloacae* GHMGHE26 (50.66 mm) was shown the highest sensitivity to GEN, but *E. fergusonii* GHMGHE46 (8 mm) was the most resistant to this antibiotic ($p<0.05$). In addition, CEF was strongly inhibited *K. pneumoniae subsp. rhinoscleromatis* GHMGHE27 (31.00 mm) while having no effect on the *Escherichia* strains ($p<0.05$). Against AMX, *E. asburiae* GHMGHE22 (55.00 mm) was the most sensitive isolate but *E. cloacae*, *K. pneumonia*, and *E. fergusonii* (0.00 mm) were the most resistant ($p<0.05$). In contrast to the weakest inhibitory effect on *E. fergusonii* GHMGHE46 (0.00 mm), CYP strongly inhibited the growth of *K. pneumoniae* GHMGHE24 (30.66 mm), ($p<0.05$).

Antibacterial Ability: The antibacterial ability of the TPBs is shown in Table 3. They had the specific ABA against *E. coli* ATCC 13706, *S. aureus* ATCC 9027, and *P. aeruginosa* ATCC9027 (Fig. 1g, 1h, 1i). The highest antibacterial potential was observed by isolate *K. pneumoniae* subsp. *rhinoscleromatis* GHMGHE27 (32 mm) against *E. coli* ATCC 13706, isolate *Escherichia coli* GHMGHE47 (40 mm) against *S. aureus* ATCC 9027, and isolate *E. fergusonii* GHMGHE48 (25 mm) against *P. aeruginosa* ATCC9027, respectively ($p<0.05$).

Table 2- The Enzyme Potentials and Growth of Tannin-producing Bacteria (TPB) on Bile Agar Media, and the Viable Cells (log CFU ml^{-1}) of TPBs Incubated at pH of 2.5 and 7

isolate	*EP			Bile (%)			**SR	
	cellulase	protease	amylase	0.5	1	2	pH: 7	pH: 2.5
K. michiganensis GHMGHE21	+	+	-	+	+	+	9.47^{a}	9.08
E. asburiae GHMGHE22	+	-	-	+	+	+	9.33^{ab}	9.21;
E. cloacae subsp dissolvens GHMGHE23	+	+	-	+	+	+	9.43^{ab}	9.20
K. pneumoniae GHMGHE24	+	-	+	+	+	+	9.46^{a}	9.00
E. cloacae GHMGHE25	+	+	-	+	+	+	9.07^{b}	9.11
E. cloacae GHMGHE26	+	-	-	+	+	+	9.22^{ab}	9.00
K. subsp. rhinoscleromatis GHMGHE27	+	+	-	+	+	+	9.48^{a}	9.16
E. fergusonii GHMGHE28	+	+	-	+	+	+	9.15^{ab}	9.00
E. fergusonii GHMGHE43	+	-	-	+	+	+	9.48^{a}	9.12
E. fergusonii GHMGHE44	+	+	+	+	+	+	9.18^{ab}	9.01
E. fergusonii GHMGHE45	+	-	-	+	+	+	9.35^{ab}	9.04
E. fergusonii GHMGHE46	+	+	-	+	+	+	9.32^{ab}	9.11
E. coli GHMGHE47	+	+	+	+	+	+	9.13^{ab}	9.00
E. fergusonii GHMGHE48	+	-	-	+	+	+	9.46^{a}	9.22
E. coli GHMGHE49	+	-	+	+	+	+	9.47^{a}	9.08
SEM							0.07	0.10
p-value							0.01	0.97

*Enzyme potential; **SR: survival rate; ***All means with different Latin alphabets have been statistically categorized into different groups ($p<0.05$).

Table 3- The Antibiogram and Antibacterial Activity of TPBs based on the Diameter of the Inhibition Zone (mm)

Isolate[1]	*AD				**SPS		
	***GEN 10 µg	CEF 5 µg	AMX 25 µg	CYP 5 µg	*E. Coli*	*S. Aureus*	*P. aeruginosa*
K. michiganensis GHMGHE21	45.33^{b}(S)[3]	6.33^{f}(R)	38.66^{b}(S)	17.66^{d}(S)	18.00^{b}	18.00^{ce}	12.00^{cd}
E. asburiae GHMGHE22	45.00^{b}(S)	26.33^{b}(S)	55.00^{a}(S)	12.00^{ef}(R)	6.00^{c}	22.00	12.00^{cd}
E. cloacae subsp dissolvens GHMGHE23	41.33^{c}(S)	0.00^{g}(R)	0.00^{g}(R)	10.00^{f}(R)	6.00^{c}	0.00^{h}	10.00^{ed}
K. pneumoniae GHMGHE24	30.00^{e}(S)	0.00^{g}(R)	0.00^{g}(R)	30.66^{a}(S)	6.00^{c}	20.66^{c}	0.00^{f}
E. cloacae GHMGHE25	21.00^{f}(S)	30.00^{a}(S)	0.00^{g}(R)	16.66^{de}(I)	10.00^{c}	33.33^{b}	10.00^{ed}
E. cloacae GHMGHE26	50.66^{a}(S)	18.00^{c}(I)	16.66^{d}(I)	10.00^{f}(R)	21.66^{b}	14.00^{ef}	15.00^{c}
K. pneumoniae subsp. rhinoscleromatis GHMGHE27	39.33^{c}(S)	31.00^{a}(S)	20.33^{c}(S)	16.00^{de}(I)	32.66^{a}	15.66^{ef}	20.00^{b}
E. fergusonii GHMGHE28	33.66^{d}(S)	0.00^{g}(R)	7.00^{f}(R)	16.00^{de}(I)	10.00^{c}	0.00^{h}	8.00^{e}
E. fergusonii GHMGHE43	11.66^{hi}(R)	8.00^{ef}(R)	8.00^{f}(R)	25.66^{bc}(S)	0.00^{d}	30.00^{b}	21.00^{b}
E. fergusonii GHMGHE44	21.33^{f}(S)	0.00^{g}(R)	16.33^{d}(I)	25.00^{c}(S)	0.00^{d}	8.00^{g}	20.00^{b}
E. fergusonii GHMGHE45	14.00^{h}(I)	10.66^{de}(R)	0.00^{g}(R)	30.00^{ab}(S)	0.00^{d}	20.00^{c}	0.00^{f}
E. fergusonii GHMGHE46	8.00^{ij}(R)	0.00^{g}(R)	12.66^{e}(R)	0.00^{g}(R)	0.00^{d}	20.00^{c}	0.00^{f}
E. coli GHMGHE47	9.33^{ij}(R)	0.00^{g}(R)	15.33^{de}(I)	15.00^{def}(R)	0.00^{d}	40.66^{a}	0.00^{f}
E. fergusonii GHMGHE48	17.00^{g}(S)	12.00^{d}(R)	14.00^{de}(I)	16.00^{de}(I)	0.00^{d}	15.00^{ef}	24.66^{a}
E. coli GHMGHE49	22.00^{f}(S)	16.33^{c}(I)	13.66^{de}(R)	16.00^{de}(I)	0.00^{d}	12.00^{fg}	15.33^{c}
SEM	0.55	0.62	0.62	0.92	0.81	0.96	0.68
*****p-value*	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

*AD: Antibiotic disc; **SPS: Standard pathogen strains; ***GEN 10 µg: gentamicin, CEF 5 µg; cefixime, AMX 25 µg: amoxicillin, CYP 5 µg: ciprofloxacin; **** S: sensitive, I: intermediate, and R: resistant; *****All means with different Latin alphabets have statistically categorized in different group ($p<0.05$).

Discussion and Conclusions

The improper use of antibiotics in the livestock industry has increased global concerns about human health. The resistant bacteria inactivate antibiotics, by producing enzymes, reducing or restricting their entry into the target site of bacteria, mutating the cellular functions, and/or altering the target site (30). Thus, exploring alternatives for well-recognized antibiotics is considered a necessary need in the livestock industry. The current study reported a total of 15 TPBs that

belonged to *Enterobacteriaceae* and some of them were indole producers. Due to the production of indole, these bacteria can contribute to the unpleasant smell of rumen contents (31). In the current study, the highest amount of produced tannase was equal to 9.39 U ml^{-1}. Similar studies reported the TPBs such as *Klebsiella* with 0.5 U ml^{-1} TAA, *Bacillus subtilis* with 0.6 U ml^{-1} TAA, *Lactobacillus rhamnosus* with 0.031 U ml^{-1} TAA, *L. bulgaricus* 0.002 U ml^{-1} TAA, and *Lactobacillus* sp. with 0.85 U gds^{-1} TAA (32-34). Kohl et al. (35) isolated different strains of *Escherichia* with remarkably similar TAA. Gheibipour et al. (6) found *E. fergusonii* (8.59 U ml^{-1}) and *E. coli* (1.30 U ml^{-1}) that had diverse TAA. It was reported that TAA varied considerably between bacterial species and even isolates of the same species (35). In addition to isolation sources, many physicochemical factors can affect TAA, including temperature, aeration, agitation speed, source of carbon, and pH level (35). In line with current findings, tannase producer-Gram-negative rods in the rumen of wild ruminants were observed (18). Brooker et al. (36) screened the tannin-degrading *Streptococcus gallolyticus* from the rumen of wild goats. Also, Beniwal et al. (37) and Singh et al. (38) isolated *E. cloacae* and *E. ludwigii* with high TAA from the rumen of migratory goats. In another study, *K. pneumonia* and *Acintobacter bumanii* with a potent ability to produce tannase were found in the rumen of deer (6). The rumen flora is the richest source of microbial enzymes. It is well documented that the ruminal bacterial enzymes can degrade tannic acid and natural tannins, and are a feature of foregut fermentation that is widely observed among tannin-consuming ruminants (38). To overcome dietary challenges and receive a satisfactory result, the cellulase, amylase, and protease potentials of TPBs were studied and the current TPBs were shown different enzyme abilities. As previously reported, cellulase, amylase, protease, and lipase activities of rumen were obseved (39). In ruminant, the presence of various classes of enzymes for pre-digestion of feed components is required and enzyme activity of rumen microbes shift according to the feed composition (39).

In addition to diverse enzyme potentials, animal feed additives must be resistant to acid and bile salts and remain active in the gut environment. In this study, TPBs were active at low pH and high bile that could be due to the compatibility of the physicochemical condition of experiments with the source of bacterial isolation. The origin of isolation is a fundamental element that directly influences the effectiveness of AFA in actual conditions, and host-derived additives are far superior to other environments (40). Another important feature for choosing AFA is its non-pathogenic nature for the host. The current TPBs had shown mixed responses to the used antibiotics. Like these results, Nijsten et al. (41) isolated *E. coli* strains from the fecal flora of pigs that were resistant to oxytetracycline, streptomycin, and sulfamethoxazole. Also, resistance to kanamycin and susceptibility of to erythromycin, chloramphenicol, and tetracycline were reported for *Lactobacillus* strains (42). Unlike ampicillin, tetracycline, and erythromycin, the growth of *L. rhamnosus* and *L. paracasei* was completely inhibited in the presence of kanamycin (43). Many species of *Lactobacillus*, *Bacillus*, and *Enterococcus* have transferable ABR genes, while *Lactobacillus* species are present in the composition of many commercial probiotics. The presence of ABR in *B. subtilis* was frequently reported as a famous probiotic (44). A similar study found that *Enterobacter asburiae* E7, a novel potential probiotic, was resistant to some antibiotics and sensitive to others (40). In a recent study, *E. asburiae* C28 isolated from the intestine of *Carassius auratus* reduced the load of potential pathogens (45). Gram-

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negative bacteria, in particular *Enterobacteriaceae*, have acquired or selected many ABR genes. They initially emerged in *K. pneumoniae*, but are now observed in *E. coli* that lives in human and animal guts. The ABR genes of probiotic strains may not be a serious problems if they are intrinsic to chromosomes and are not transferable (46).

Another important feature for choosing a bacterium, as an AFA, is its antibacterial effects. In this study, the agar diffusion method was used to evaluate the ABA of TPBs that revealed the excellent ABA of strains against pathogen *E. coli*, *S. aureus*, and *P aeruginosa*. In a study, the probiotics, *B. subtilis*, and *B. licheniformis* strongly repressed gut pathogens by producing antimicrobial materials (47). As reported previously, *E. coli* had produced an antimicrobial compound against *S. enterica* subsp. *enterica serovar* Newport ATCC 6962 (48). Also, LAB, *Bifidobacteria* and *Bacillus* produced thermostable bacteriocins to suppress animal pathogens (49). In a study, *E. coli* Nissle 1917 was more effective than *L. rhamnosus* and *Berevibacillus laterosporus* to inhibit the enteric pathogens (50). Interestingly, strains of probiotic *E. coli* Nissle 1917 were active against *Listeria* or *Candida* (51, 52). In an *in vitro* study, *E. coli* G3/10, a component of the probiotic drug, suppressed enteropathogenic *E. coli* E2348/69 by producing microcin (53). Thus, TPBs may exclude pathogens by competing for receptor sites, absorbing nutrients, and secreting the antibacterial molecules (lactic acid, hydrogen peroxide), in particular, bacteriocins (48). As previously mentioned, gallic acid is produced due to hydrolyzing tannin has antifungal, antiviral, and antioxidant properties that can stimulate the growth of probiotic microbes and help to inhibit pathogens (34). A greater focus on examining the rumen microbiome of non-domesticated ruminants could help to identify novel commercial microbes and enzymes. So far, a wide range of Gram-positive bacteria have been introduced as common feed additives but the probiotic potential of Gram-negative bacteria has rarely been studied. So, screening of new Gram-negative species such as present isolates to enhance probiotic libraries is required. This study identified *Escherichia fergusonii* GHMGHE44 with a superior ability to degrade tannin. The isolates were active in the gut conditions, *in vitro*, and had antibacterial potentials. Therefore, current TPBs isolated from deer rumen may be excellent candidates to be used as AFA. Overall, probiotic microbes approved for animals' diets can be high safety and have no health hazards. Considering the current results and the strong inhibitory effect of tannin on the growth of Gram-positive bacteria, the identification and in-depth study of Gram-negative probiotics for use in high-tannin diets is suggested.

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Data Availability

The datasets generated during the current study are available online in the Gen Bank at NCBI database repository [https://www.ncbi.nlm.nih.gov/nuccore/?term=MZ891587:MZ891635[accn]]

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Seyyed Ehsan Ghiasi reports financial support provided by Iran National Science Foundation.

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