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Research

Isolation and Identification of Tannin-Degrading Bacteria From Goat Feces, Ruminal Fluid, and Rumen Gut

Muhammad Syafiq Suhaimi[1], Fayyadhah Asyiqin Zailani[1], Nur Farah Syuhada Mohd Zaki[1], Farizan Aris[1], Mohd Taufiq Mat Jalil[1], Nurul Aili Zakaria[1,2]*

1. Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
2. Human Genetics and Biochemistry Research Group (Hugeb), Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
*Corresponding author: nurulaili@uitm.edu.my

ABSTRACT

Tannins are toxic polyphenols present in various plants, contributing to microbial attacks and plant protection due to their astringence and bitter taste. However, high tannin inclusion in poultry diets will result in dyspepsia, hampering nutrient absorption and digestion. Interestingly, several bacteria occupying the rumen and gastrointestinal tract (GIT) of animals may tolerate tannins and degrade them by wielding tannase enzymes. The study aims to isolate and characterize potential tannin-degrading bacteria (TDB) from several ruminant specimens. The TDBs were isolated based on their tannin hydrolyzing ability on a minimal salt medium (MSM) agar complemented with 0.2% tannic acid as the sole source of carbon and energy. The maximum tannin tolerance of the isolates was characterized using increased tannin concentrations on the MSM agar plates. Furthermore, the tannase activity was also evaluated over a five-day incubation. A total of 42 tannin degraders were isolated, and 10 TDBs were chosen for further characterization based on the hydrolyzed zone produced. Molecular identification revealed the presence of *Bacillus cereus* (TDB536), *Lysinibacillus macroides* (TDB17), *Acinetobacter nosocomialis* (TDB18, 20, 23, 24, 30, 35), and *Staphylococcus saprophyticus* (TDB40). TDB17, TDB18, and TDB24 showed the highest tannic acid tolerance at 1.0%, while TDB36 and TDB40 exhibited the lowest tolerance at 0.4%. Each TDB displayed varying tannase activities, ranging from 11.56 to 42.08 U/mL over a five-day incubation period. TDB5 and TDB35 demonstrated significantly higher tannase activity on day 2 ($p<0.05$). Meanwhile, TDB23 and TDB24 showed the highest tannase on day 4 ($p<0.05$). Among the isolates, *A. nosocomialis* strain AE6 (TDB24) from feces exhibited the highest tannase activity (42.08 U/mL) and represented the best TDB. The isolated strains demonstrate their capabilities in reducing tannin's antinutritional effects in poultry feed.

Key words: *Acinetobacter strain*, identification, tannase, tannic acid, tannin-degrading bacteria

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INTRODUCTION

Tannins are a heterogeneous group of naturally found polyphenolic compounds that can be categorized into three primary forms, i.e., hydrolyzable, complex, and condensed ones, based on their chemical structure and characteristics (Smeriglio *et al*., 2017; Das *et al*., 2020; Tong *et al*., 2022). These compounds are majorly present in various plants, partaking in microbial attacks and plant protection due to their astringence and bitter taste (Govindarajan *et al*., 2016; Bhatla & Lal, 2018). Tannins also possess antimicrobial properties as they exert toxicity towards bacteria, which arises from enzyme inhibition, substrate deprivation, their effect on membrane function, and the induction of metal ion deficiency (Huang *et al*., 2018).

However, several microorganisms have been documented to thrive on tannins and utilize them as their sole source of carbon and energy via the action of tannin acyl hydrolases, also known as tannases (Narayanaswamy *et al*., 2023). It catalyzes the hydrolysis of tannins to form simpler and less complex compounds like gallic acid, glucose, and galloyl ester (Lekshmi *et al*., 2021). Tannase was discovered in 1867 by Van Teighem, which led to studies on the aspects

of tannase production. Studies revealed that tannase is an inducible enzyme that could be produced by the most potential tannase producers, such as filamentous fungi like *Aspergillus* sp. and *Penicillium* sp. via solid-state fermentation (SSF), and widely used in most industries (Aharwar & Parihar, 2018). Consequently, most studies were focused more on fungal tannases, as tannins were previously thought to be bacteriostatic (Belur & Mugeraya, 2011).

Nonetheless, despite a strong focus on the production of tannase by bacterial strains from 1990 onwards following its discovery in 1983, reports on it are still scarce (Belur and Mugeraya, 2011; Dhiman *et al*., 2021; Liu *et al*., 2022). These bacteria, identified as tannin-degrading bacteria (TDB), have been reported from various genera, including *Bacillus*, *Klebsiella*, *Lactobacillus*, *Citrobacter*, *Pediococcus*, *Pseudomonas*, *Staphylococcus*, *Corynebacterium*, *Serratia*, *Leuconostoc*, *Pantoea*, *Selenomonas*, and *Streptococcus* (Sarwat, 2014; Tahmourespour *et al*., 2016; Mohammed, 2016; Tripathi *et al*., 2016). Dhiman and Mukherjee (2020) reported that bacterial tannases might degrade natural tannin and tannic acid into gallic acid more efficiently on an industrial level compared to fungi, despite the latter demonstrating better hydrolyzable tannin degradation activity. However, a major problem with the use of fungal strains is their relatively slow growth rate and genetic complexity (Kumar *et al*., 2015). Contrastingly, bacterial strains exhibit a very high growth rate, and they can be easily manipulated at the genetic level (Beniwal *et al*., 2014).

Tannins may also be found in several common feed ingredients utilized in poultry diets, such as sorghum and barley, as they constitute one of the most abundant components in plants (Hidayat *et al*., 2021). Their presence may result in dyspepsia, reducing digestibility and nutrient absorption, thus leading to the accumulation of residual tannins, which can contribute to toxicity (Brouwer *et al*., 2018). Nevertheless, a wide range of bacteria inhabiting the rumen and gastrointestinal tract (GIT) of animals can tolerate tannins and degrade them by the action of tannase enzymes (Tahmourespour *et al*., 2016; Cipriano-Salazar *et al*., 2018). Consequently, fecal and alimentary tract samples from animals serve as viable sources for isolating TDB due to the presence of a complex microbial population in the GIT, which is predominantly regulated by nutrition (Choudhury *et al*., 2019).

This study aims to isolate and identify TDB from various ruminant specimens, i.e., from the GIT, ruminal fluid, and fecal matter. Moreover, this study highlights the activity of tannase produced by TDBs to explore their potential application in mitigating elevated tannin levels in poultry diets.

MATERIALS AND METHODS

Sampling and bacterial suspension preparation

The goat feces samples were collected from Jariah Agro Farm, Klang, Selangor (3.0371°N, 101.4859°E), while the rumen gut and ruminal fluid samples were obtained from slaughtered ruminants at the Kompleks Abatoir Shah Alam (3.057442°N, 101.519337°E). The samples were collected in a 15 mL sterile phosphate-buffered saline (PBS) solution in a universal bottle (Figure 1). They were sonicated using an ultrasonic water bath and vortexed for 10 sec to facilitate the separation of bacterial cells from the supernatant (Tahmourespour *et al*., 2017). No ethical approval was required for this as it did not involve the use of live animals, animal handling, or treatment.

Fig. 1. The collected samples of ruminant's gut, ruminal fluid, and goat feces.

Preliminary screening and isolation of TDB

TDB isolation was conducted following Balakrishnan *et al*. (2021). Aliquots of 1 mL of serially diluted

sample suspensions (10^{-1} to 10^{-7}) were plated onto minimal salt medium (MSM) agar containing K_2HPO_4 (0.5 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L), NH_4Cl (1.0 g/L), $CaCl_2$ (0.1 g/L), and 3% agar with 0.2% tannic acid. The plates were incubated at 37°C for up to 48 hr to observe the hydrolysis zone. The use of tannic acid as the sole source of carbon and energy was based according to Brinda Lakshmi *et al*. (2013). The positive isolates were preserved in glycerol and kept at -20°C for further analysis.

In-vitro morphological and biochemical characterization of TDB

The TDB isolates were subjected to characterization based on their colonial morphology and biochemical properties using several biochemical tests.

Cell Morphology

A thin smear was prepared by transferring a colony of bacteria to a droplet of distilled water on a sterile glass slide and fixed by heating. The smear was covered with 1% crystal violet, followed by Gram iodine, ethanol, and safranin red counterstain. Each step was completed with a rinse in between. The slide was then observed under the microscope for characterization of the isolates based on Bergey's Manual of Systematics of Archaea and Bacteria (BMSAB) by Whitman (2015).

Catalase test

A colony of bacteria was transferred to a droplet of 3% hydrogen peroxide placed on a clean glass slide. The presence of bubbles indicates a catalase-positive isolate (Tahmourespour *et al*., 2016).

Oxidase test

A single droplet of Kovac's oxidase reagent was applied to the Whatman No. 1 filter paper. A colony of bacteria was carefully transferred and gently applied to the oxidase reagent. The presence of dark purple is interpreted as a positive result (Tahmourespour *et al*., 2016).

Identification using 16S rRNA molecular technique

The selected isolates were identified by sequencing the 16S rRNA gene. Isolates were grown in nutrient broth at 37°C overnight, and the genomic DNA was extracted according to standard protocol using the NucleoSpin Tissue Kit (MACHEREY-NAGEL). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) in a Bio-Rad Thermal Cycler (Model T100™). The PCR mixture consisted of 1× Green Buffer Dye, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 1 U Taq DNA polymerase, 0.25 µM of universal primers 27F and 1492R with 1 µL of genomic DNA in a total volume of 50 µL. The thermocycling conditions were as follows: 95°C for 3 min (initial degradation), 30 amplification cycles (denaturation at 95°C for 30 sec, annealing at 52°C for 20 sec, and extension at 72°C for 1 min 40 sec) and a final extension at 72°C for 5 min (Kaczmarek-Szczepańska, 2021). The PCR products were electrophoresed on 1% agarose gel stained with GelRed nucleic acid stain and were Sanger sequenced at Bio Basic Asia Pacific Pte Ltd (Singapore). The sequences obtained were analyzed using the BLASTn suite at the National Center for Biotechnology Information (NCBI) server (https://blast.ncbi.nlm.nih.gov). The phylogenetic tree of sequences was constructed using the MEGA11 software.

Evaluation of the tannin tolerance of TDB

The tolerance of the TDB isolates to tannic acid was assessed according to Tahmourespour *et al*. (2017) with slight modifications. MSM agar plates containing increasing concentrations of tannic acid (0.2%, 0.4%, 0.6%, 0.8% & 1.0%) were used to determine the maximum tolerance. Wells were made in the agar to facilitate the inoculation and diffusion of bacterial cells. Before inoculation, the optical density of the bacterial suspension was adjusted within the range of 0.95–1.0 at 600 nm (10^8 CFU/mL) by spectrophotometry. The plates were incubated at 37°C for up to 48 hr.

Tannase enzyme assay

The tannase assay for TDB followed the method by Balakrishnan *et al*. (2021). An aliquot of 1 mL of TDB culture was added to 100 mL MSM broth containing 0.2% tannic acid at pH 7.0. The mixture was incubated at 37°C with agitation at 220 rpm for 5 days. Samples were collected every 24 hr throughout 120 hr for tannase production estimation. Subsequently, the sample was centrifuged at 4000 rpm at 4°C for 10 min, and the supernatant was used for the tannase assay (Shakir *et al*., 2022a).

The tannase assay utilized a chromogen synthesis method with gallic acid and rhodanine (Srivastava

and Kar, 2010). In the procedure, 0.25 mL of 0.01 M methyl gallate in a 0.05 M citrate buffer (pH 5.0) was mixed with 0.25 mL of extracellular enzyme, followed by a 10 min incubation at 30°C. After incubation, 0.3 mL of 0.667% (w/v) methanolic rhodanine was added, and after 5 min, 0.2 mL of 0.5 M potassium hydroxide (KOH) was introduced. A control experiment reversed the enzyme and KOH order. A volume of 4.0 mL of water was added to the reaction mixture and incubated for 10 min at 30°C before absorbance measurement at 520 nm. A blank with mineral salt medium and tannin served as a reference. One unit of tannase released 1 mole of gallic acid in 1 min.

RESULTS

Isolation and identification of tannin-degrading bacteria (TDB)

The isolation of TDB was performed by observing the appearance of a clear hydrolysis zone on MSM agar, with tannic acid acting as the sole carbon and energy source. A total of 42 microorganisms were obtained from three samples: ruminal fluid (31 isolates), ruminal gut (8 isolates), and feces (3 isolates). These isolates demonstrate a clear zone of hydrolysis on the plates, confirming their ability to degrade tannin (Figure 2).

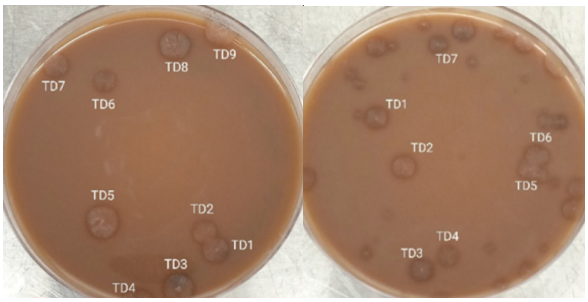

Fig. 2. Preliminary screening of TDB on MSM agar with 0.2% tannic acid.

Visual assessment of the hydrolysis zone after 48 hr varied from 0.3 to 2.5 cm. Figure 3 depicts the percentage distribution of isolates according to the diameter of the hydrolysis zones. A higher proportion of microbes with smaller hydrolysis zone diameters between 0.1 and 0.5 cm is observed in ruminal fluid (52%) compared to ruminal gut (15%) and feces (5%). As the hydrolysis zone increases, the percentage of isolates decreases. Several TDB isolates from ruminal fluid exhibit larger hydrolysis zones, measuring between 1.0 and 2.5 cm.

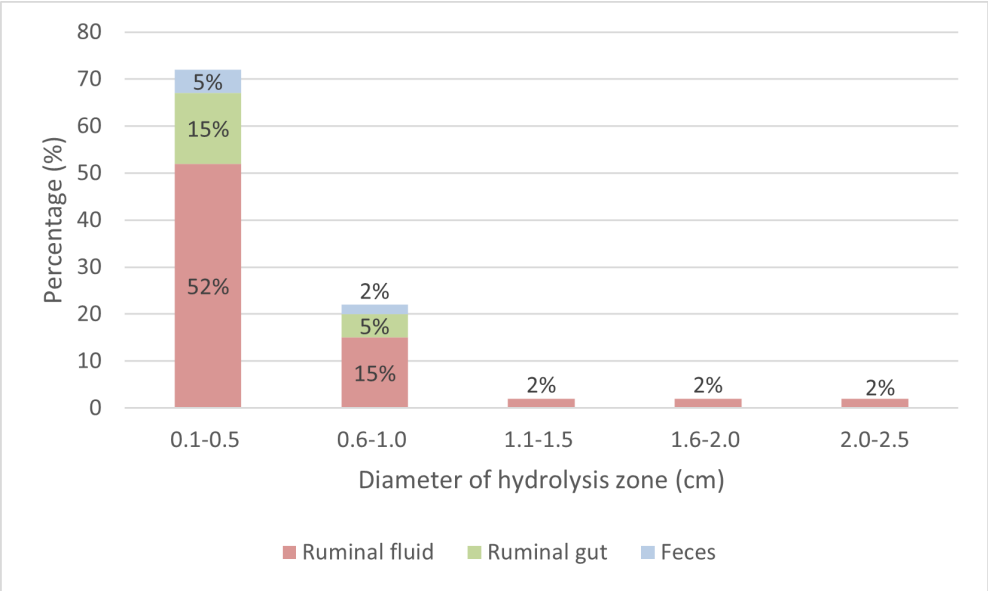

Fig. 3. Percentage distribution of isolates based on the diameter of the hydrolysis zones on MSM agar.

Based on the diameter of the hydrolysis zones, ten isolates (TDB 5, 17, 18, 20, 23, 24, 30, 35, 36 & 40) were selected for morphological and biochemical characterization (Table 1). Six isolates are Gram-negative, and four are Gram-positive. The isolates are also catalase-positive and oxidase-negative. The tests were consistent with the *Bacillus*, *Lysinibacillus*, *Acinetobacter*, and *Staphylococcus* genera.

Table 1. The results of Gram staining and biochemical tests of the selected ten TDB isolates.

Sample source	TDB	Gram	Catalase	Oxidase	Expected genus
Ruminal gut	5	+	+	-	*Bacillus*
Ruminal fluid	17	+	+	-	*Lysinibacillus*
Ruminal fluid	18	-	+	-	*Acinetobacter*
Ruminal fluid	20	-	+	-	*Acinetobacter*
Ruminal fluid	23	-	+	-	*Acinetobacter*
Feces	24	-	+	-	*Acinetobacter*
Ruminal fluid	30	-	+	-	*Acinetobacter*
Ruminal fluid	35	-	+	-	*Acinetobacter*
Ruminal fluid	36	+	+	-	*Bacillus*
Ruminal fluid	40	+	+	-	*Staphylococcus*

Upon comparison of the 16S rRNA gene sequences (strains TDB 5, 17, 18, 20, 23, 24, 30, 35, 36 & 40) with the sequences in GenBank, all strains exhibit up to 99% similarity to four distinct bacterial genera: *Bacillus* (2), *Lysinibacillus* (1), *Acinetobacter* (6), and *Staphylococcus* (1) (Table 2). TDB5 and TDB36 demonstrated over 99.5% similarity to *Bacillus cereus* LB073 and RJ06B strains. Similarly, TDB17 exhibits similarity (99.91%) to *Lysinibacillus macroides* strain CS26. Six of the isolates (TDB 18, 20, 23, 24, 30 & 35) exhibit the highest similarity (>99%) to *Acinetobacter nosocomialis* 4723 and AE6 strains. Meanwhile, TDB40 exhibits a 100% similarity to *Staphylococcus saprophyticus* strain YSY1-3, implying a remarkably conserved genetic composition and a likely shared lineage. This finding has underscored the taxonomic relationships and genomic agreements among microbial isolates, offering insights into their evolutionary connections and potential functional commonalities. These insights contribute essential data that enrich the overall understanding of microbial diversity and taxonomy within the study's scope. The phylogenetic tree was constructed using a neighbor-joining method, enabling a comparison between each bacterial strain, as depicted in Figure 4.

Table 2. 16S rRNA gene sequencing results of ten TDBs isolated from feces, ruminal fluid, and rumen's gut.

Sample source	Isolate	Closest Species	Similarity (%)	Length (bp)	Accession Number
Ruminal gut	TDB5	*Bacillus cereus* strain LB073	99.68	1121	KY622536
Ruminal fluid	TDB17	*Lysinibacillus macroides* strain CS26	99.91	1457	KR780381
Ruminal fluid	TDB18	*Acinetobacter nosocomialis* strain 4723	99.40	1395	MH084921
Ruminal fluid	TDB20	*Acinetobacter nosocomialis* strain AE6	100	1472	MT540255
Ruminal fluid	TDB23	*Acinetobacter nosocomialis* strain AE6	99.91	1472	MT540255
Feces	TDB24	*Acinetobacter nosocomialis* strain AE6	99.92	1472	MT540255
Ruminal fluid	TDB30	*Acinetobacter nosocomialis* strain AE6	99.91	1472	MT540255
Ruminal fluid	TDB35	*Acinetobacter nosocomialis* strain AE6	100	1472	MT540255
Ruminal fluid	TDB36	*Bacillus cereus* strain RJ06	99.54	1432	KT718054
Ruminal fluid	TDB40	*Staphylococcus saprophyticus* strain YSY1-3	100	1444	GU197531

Maximum tannin tolerance level of the isolates

The ten TDB isolates that showed positive outcomes in the preliminary screening demonstrated maximum tolerance on MSM agar with tannin concentrations ranging from 0.2% to 1.0%. The appearance of hydrolysis zones on the plates following the introduction of $FeCl_2$ solution into the medium at 48 h of incubation signifies that all isolates can tolerate tannin concentrations up to 0.4% (Figure 5).

The diameter of the hydrolysis zones produced decreases as the tannin concentration increases. Among the isolates, TDB36 (*B. cereus* strain RJ06) and TDB40 (*S. saprophyticus* strain YSY1-3) are the least tolerant, with a maximum tannin concentration of only 0.4%. Subsequently, TDB5 and TDB35 (*B. cereus* strain LB073 and *A. nosocomialis* strain AE6) showed moderate levels of tannin tolerance at 0.6%. Conversely, TDB23 (*A. nosocomialis* strain AE6) tolerated 0.8% tannin. Meanwhile, TDB17 (*L. macroides* strain CS26), TDB18 (*A. nosocomialis* strain 4723), and TDB24 (*A. nosocomialis* strain AE6) exhibit the highest tolerance of up to 1.0% (Figure 6).

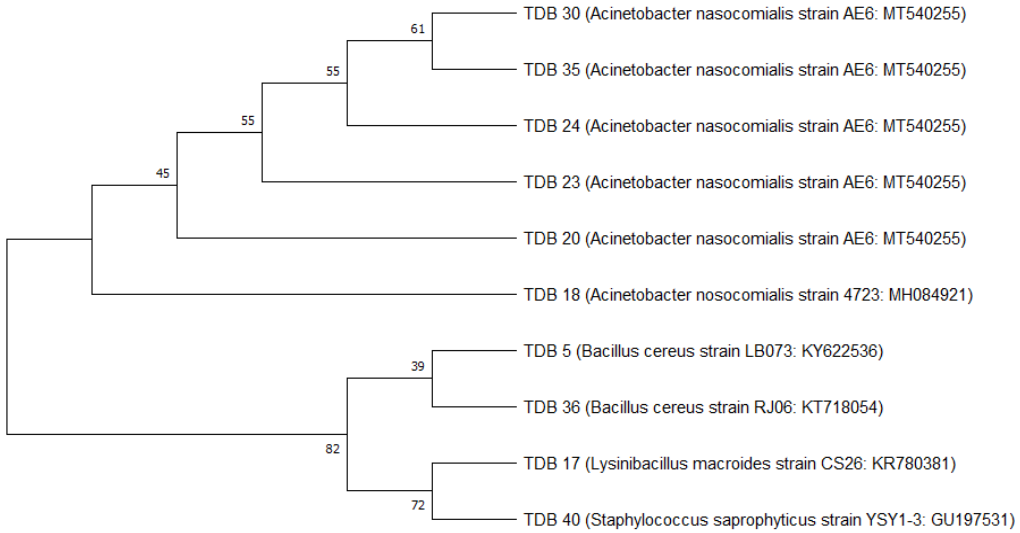

Fig. 4. The phylogenetic tree was constructed using the neighbor-joining (NJ) method, which illustrates the evolutionary relationship among all strains based on their 16S rRNA gene sequences.

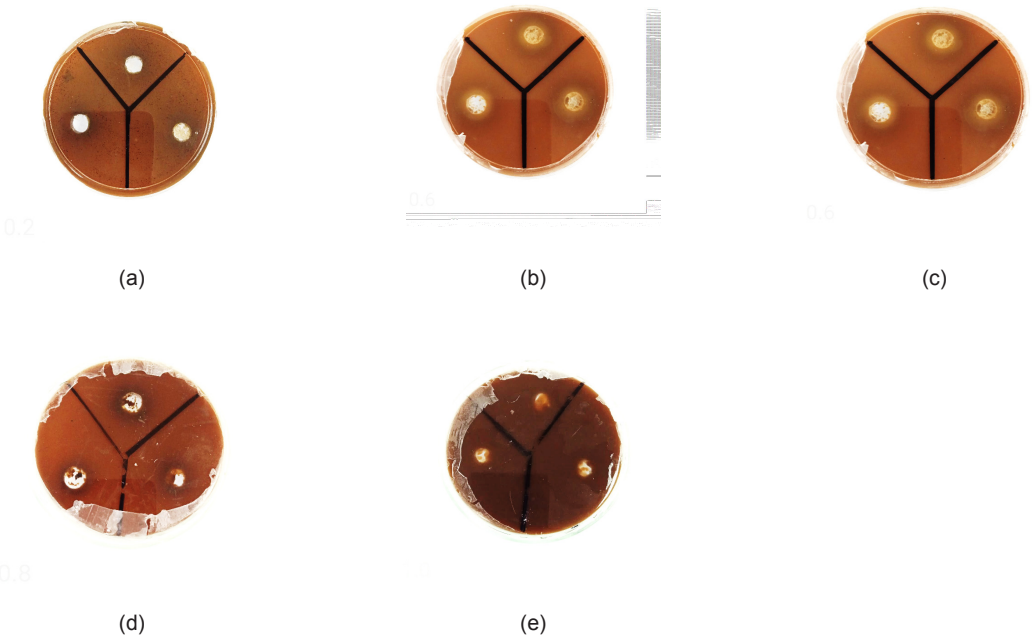

Fig. 5. Maximum tannin tolerance of the isolates against various concentrations of tannin: (a) 0.2%, (b) 0.4%, (c) 0.6%, (d) 0.8%, and (e) 1.0%.

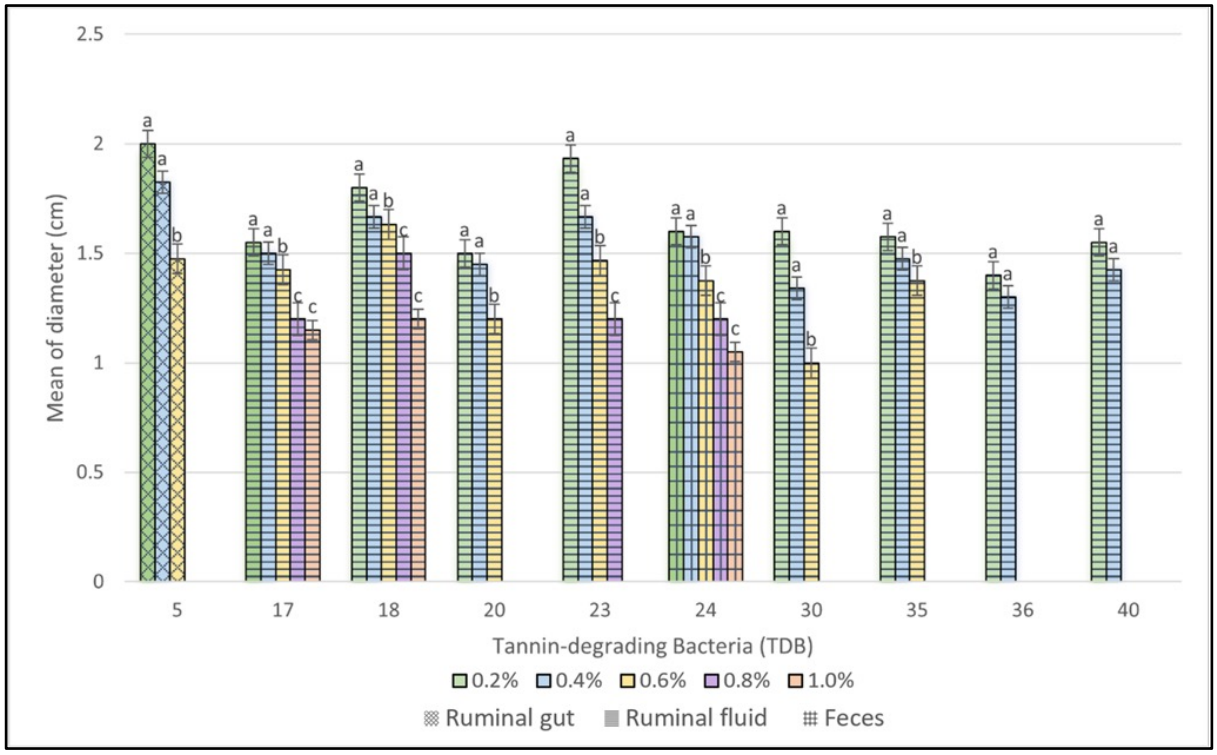

Fig. 6. Graph of the mean of diameter (cm) against concentration of tannin (%).

Tannase enzyme assay

All isolates were then assessed for their tannase activity in a minimal medium broth containing 0.2% tannic acid at pH 7.0 to determine the most effective tannase producer. Figure 7 represents the effect of the incubation period on tannase production by each isolate.

For the 5-day incubation experiment, the tannase activity showed a significant difference (p<0.05) in increments ranging from the lowest of 13.64 U/mL to the highest of 42.08 U/mL. TDB5 and TDB35 showed the highest tannase activity on day 2, with 31.70 U/mL and 25.82 U/mL. Meanwhile, most of the isolates displayed the highest tannase activity on day 3. In contrast, TDB23 and TDB24 demonstrate the highest activity on day 4, with 35.72 and 42.08 U/mL. Particularly, TDB24 exhibits the highest tannase activity at the optimum level than other TDBs (p<0.05). All isolates showed a subsequent decrease in enzyme activity, varying from a minimum of 11.56 U/mL to a maximum of 28.31 U/mL after reaching their peak.

DISCUSSIONS

Ruminants, herbivores that consume several plant components such as leaves, bark, fruits, and seeds, encounter naturally occurring polyphenolic compounds known as tannins in their diet (Lall *et al*., 2018). These tannins resist complete degradation and absorption in the digestive systems of the ruminants (Besharati *et al*., 2022). Consequently, the GIT serves as a reservoir for numerous microbial populations involved in tannin breakdown (Schmidt and Zsedely, 2011). Studies have successfully isolated and identified tannin-degrading microorganisms from various sources (Kumar *et al*., 2014; Tahmourespour *et al*., 2017; Gheibipour *et al*., 2022). Tannin-degrading microorganisms were successfully isolated from sources such as the ruminal fluid, ruminal gut, and feces, indicating their potential role in tannin degradation within the digestive system of ruminants. This isolation can be attributed to a poultry diet rich in phenolic compounds fed to the ruminants.

Previous studies have reported differences in microbial diversity between the rumen and feces of ruminants (de Oliveira *et al*., 2013; Durso *et al*., 2017; Lourenco *et al*., 2020). Researchers have revealed a greater microbial diversity in the rumen compared to the fecal material, which aligns with the findings of the present study. This phenomenon is ecologically rational, given that the greater availability of nutrients from the feedstuffs in the rumen would be broken down and absorbed before reaching the large intestine (Lourenco *et al*., 2020). Several gastrointestinal microorganisms isolated from adapted

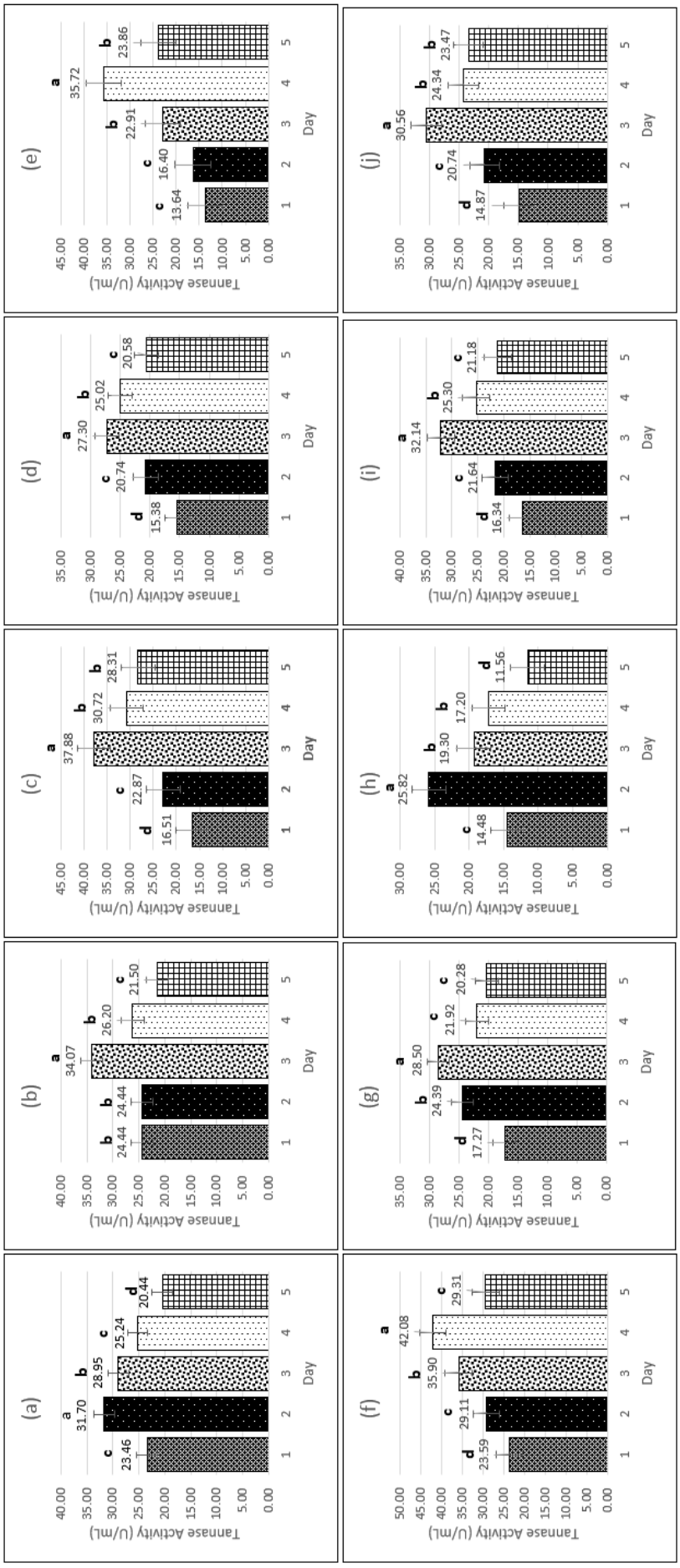

Fig. 7. Tannase production of ten isolates: (a) TDB5, (b) TDB17, (c) TDB18, (d) TDB20, (e) TDB23, (f) TDB24, (g) TDB30, (h) TDB35, (i) TDB36, and (j) TDB40. Values differ significantly between groups at $p<0.05$.

domestic and wild animals, such as sheep, goats, cattle, and deer, have been shown to play a significant role in obtaining dietary proteins from a tannin-rich diet (Goel *et al*., 2015).

Tannin-degrading microorganisms typically exhibit clear zones around their colonies when grown on a medium containing tannin, indicating enzymatic activity. This activity is likely due to the synthesis of tannase, an enzyme responsible for degrading tannins within the agar (Unban *et al*., 2020). Previous studies have demonstrated the ability of isolated microorganisms, including fungi and bacteria, on tannic acid hydrolyzation. Brahmbhatt *et al*. (2014) reported that of 20 microorganisms screened, 7 fungal isolates and 13 bacterial cultures from soil samples of tea waste dump sites and agro-residue waste sites produced tannases, with hydrolysis zone diameters ranging from 1.5 to 4.8 cm. Similarly, this study disregarded several of the 42 initial isolates due to their fungal morphology, focusing only on bacteria for subsequent characterization and assays.

Molecular identification methods revealed that the ten TDBs were identified as *A. nosocomialis*, *L. macroides*, *B. cereus*, and *S. saprophyticus*. Most of these identified strains were first reported as tannin degraders in the ruminants throughout the studies on TDB. *Acinetobacter* strains with the ability to hydrolyze tannic acid were reported by Mohammadabadi *et al*. (2021) from deer gut. *A. nosocomialis* is known as part of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex (Knight *et al*., 2018). In addition, *A. baumannii* was also found in an intensive care unit (ICU) burn, producing tannase, as mentioned by Abdulshaheed *et al*. (2023). *L. macroides* demonstrated the capability to produce tannase, which corresponded to its natural degradation tendencies (Haldar & Nazareth, 2018). While detailed experiments on this aspect were lacking in earlier studies, multiple investigations have been reported on the bacterium, particularly regarding its degradation of chromium (VI) in wastewater and polypropylene film (Hernandez-Pena *et al*., 2021; Andriani *et al*., 2022). Selvaraj *et al*. (2021) identified *B. cereus* M1GT in the GIT of goats, highlighting its potential for tannase production. Meanwhile, Govindrajan *et al*. (2016) observed a similar type of bacteria in the gut of *Gryllotalpa krishnani* that produced high levels of tannase activity in plate assays. Shakir *et al*. (2022b) and Govindrajan *et al*. (2016) have confirmed the ability of *Bacillus subtilis*, isolated from both fish and insect guts, to produce tannase. Likewise, Noguchi *et al*. (2007) characterized tannases for the first time from *Staphylococcus lugdunensis* in advanced-stage colon cancer.

Nevertheless, despite the ability of the selected bacterial isolates to degrade and detoxify 0.2% tannic acid, they had to adapt to high concentrations of tannic acid in the medium. Previous studies have utilized a solution containing $FeCl_2$ to amplify the visibility of the transparent area resulting from tannin degradation in the medium (Tahmourespour *et al*., 2016). However, this study presents contrasting results compared to previous research, suggesting that the introduction of the solution may impede the visual identification of the zone. The observed phenomenon of the clear zone undergoing a color change to brown within a 24-hr incubation period is ascribed to the process of tannic acid oxidation after the degradation of the tannin-protein complex, as reported by Susan and Madhan (2021). Cipriano-Salazar *et al*. (2018) demonstrated that bacterial isolates from adult sheep showed tolerance to 2% tannin, equivalent to 0.63 and 1.25 mg/L of tannic acid. However, Tahmourespour *et al*. (2016) observed that *Klebsiella* sp. had a maximum tolerable tannin concentration of 0.05% to 1.6% before a tannin-rich diet, while Unban *et al*. (2020) reported that *Bacillus* sp. had a tannin tolerance of 0.5%. Meanwhile, Rungsirivanich and Thongwai (2020) identified *Bacillus siamensis* with a tannin tolerance of 1.0%. Although the concept of tannin being anti-nutritional or toxic to ruminants has been well documented (Tahmourespour *et al*., 2016; Yuan *et al*., 2020), the isolates in this study did not suffer from any toxicity, suggesting they may have developed adaptive mechanisms to overcome the negative effects of tannin, enabling them to metabolize or break down these substances. Hence, this adaptation ensures their survival in tannin-rich environments and derives nutrients from tanniniferous diets as a sole carbon and energy source (Tahmourespour *et al*., 2016).

According to the literature, microbial cells may employ several strategies to counteract the inhibitory effects of tanniferous feeds (Goel *et al*., 2005). Several microorganisms have been identified to produce tannase, an enzyme with esterase and depsidase activities. This enzyme specifically hydrolyzes ester and depside bonds in hydrolyzed tannins, releasing glucose and gallic acid, thereby diminishing tannin's protein-binding properties and facilitating their metabolism into less harmful substances (Lima *et al*., 2014). Additionally, the microorganisms actively produce tannin-detoxifying enzymes besides developing alternative biologically economical targets for tannins. For example, proline-rich proteins in the saliva and the incorporation of tannin-binding agents like polyvinylpyrrolidone and polyethylene glycol (PEG) can be incorporated to deactivate tannins (Besharati *et al*., 2022). Moreover, microorganisms secrete extracellular polysaccharides (EPS), forming a protective network of fibrils that separates the microbial cell wall from reactive tannins, thereby reducing their inhibitory effects on microbial growth (Roy *et al*.,

2018). Finally, modifications in the gut environment, such as changes in gut pH, can mitigate the toxic effects of tannins, while some bacteria modify their cell membranes to shield key membrane proteins from tannin binding, aiding in overcoming tannin toxicity (Xu *et al*., 2023).

The production of many bacterial tannases is induced by the tannic acid substrate. Similarly, all TDB strains in this study produced varying amounts of tannase in the presence of 0.2% tannic acid in MSM broth. Tannase serves to hydrolyze the galloyl ester linkage, liberating gallic acid, which serves as an indicator for evaluating tannase activity (Jiménez *et al*., 2014). Thus, the use of methanolic rhodanine in the reaction provides a particular approach for determining gallic acid as it selectively interacts with gallic acid and not with galloyl esters from other phenolic compounds (Wang *et al*., 2018). Moreover, it serves a dual purpose of halting the enzymatic reaction and providing the complexing agent for chromogen formation in an enzymatic reaction (Sharma *et al*., 2000). However, utilizing commercial tannic acid as the substrate in the rhodanine assay may yield high absorbance values due to the presence of a small amount of free gallic acid in the preparation (Rodríguez *et al*., 2008).

The production of tannase by TDB is supported by Abdulshaheed *et al*. (2023), who reported that *A. baumannii* gave the highest yields of tannase (26.46 mg/mL) on day 3 of incubation. The capability of *Acinetobacter* spp. as a tannase producer was only discovered in 2021 by Mohammadabadi *et al*. (2021); hence, no other detailed information has been provided. Given the limited information on tannase activity within the *Lysinibacillus* genus, a comparison was made with the *Bacillus* genus since both genera belong to the *Firmicutes* family, prompting a comparative analysis. The *Bacillus* genera generally have an optimum activity ranging from day 1 to day 3 (Tripathi *et al*., 2016; Unban *et al*., 2020; Jebur, 2020). A study by Lekshmi *et al*. (2020) is aligned with the current study, in which *Bacillus velezensis* TA3 isolated from the saltpan of soil samples was found to be optimal after day 2 of incubation with 2 U/g and declined on day 3. Nevertheless, *Bacillus licheniformis* isolated from Iraqi soil produced a maximum tannase concentration of 76.8 U/mL on day 3 (Jebur, 2020). Likewise, Tripathi *et al*. (2016) displayed that *Bacillus megaterium* showed the highest activity on day 3 of inoculation, reaching 10.77 U/mL/min, which subsequently decreased and remained stable thereafter. Meanwhile, *Bacillus gottheilii* M2S2, isolated from tannery effluent soil, exhibited optimized activity on day 1 (Subbalaxmi and Murthy, 2016). Additionally, *Staphylococcus aureus* recorded the highest tannase production on day 1 (Isah *et al*., 2017). Other than that, tannin-degrading *S. lugdunensis* associated with colon cancer was incubated for 4 days to assess tannase production for the characterization of a novel tannase gene (Noguchi *et al*., 2007).

Notably, the enzyme produced in the MSM broth directly correlates with the organism's growth. It indicates that the accumulation of extracellular enzymes is on par with the rapid expansion of cell number. All TDB strains from the ruminants produced the maximum enzyme concentration at their exponential phase of growth, at least on day 2 and the most on day 4. The enzyme activity was reduced when it entered the stationary phase (Jebur, 2020). Moreover, the decline in tannase activity could be due to the lack of nutrients in the culture medium, which hinders sustained enzyme function and reduces enzyme production, impacting the efficiency of tannin breakdown (Kumar *et al*., 2016; Kathuria *et al*., 2018; Noor and Al-Bayyar, 2018). Additionally, environmental factors such as temperature and pH may also influence these events (Jiménez *et al*., 2014; Abdulshaheed *et al*., 2023). Understanding the dynamics is crucial for optimizing tannin degradation processes in various applications, including animal feed and wastewater treatment (Tripathi *et al*., 2016; Sutaoney *et al*., 2023; Saad *et al*., 2023).

Most previous studies on TDB demonstrated the *Acinetobacter* genera as the most identified strain. This group is known to thrive in diverse environments, including water, soil, food, and wastewater (Ahuatzin-Flores *et al*., 2024). Some strains are associated solely with plants, animals, or insects (Rokhbakhsh-Zamin *et al*., 2011; Jayamani *et al*., 2015; Wareth *et al*., 2019). Yet, these *Acinetobacter species*, including *A. baumannii*, *A. calcoaceticus*, and *Acinetobacter lwoffii*, are rare in the healthy human gut microbiome and animals (Cekanaviciute *et al*., 2017). Nonetheless, some studies have isolated those species from the GITs of humans and animals, particularly in cases of infection or colonization. In such instances, the *Acinetobacter* species are often considered opportunistic pathogens capable of causing infections in individuals with compromised immune systems or underlying health conditions (Wong *et al*., 2017). Therefore, the presence of tannins in poultry diets, which is known to induce gastrointestinal discomfort and impair nutrient absorption, has led to the confirmation that TDB belongs to the *Acinetobacter* sp. that colonizes the rumen for tannin degradation.

Moreover, this genus is prominent as a TDB and is characterized as Gram-negative bacteria. The bacterial strain type is reported to be one of the factors impacting bacterial growth and metabolism of polyphenols (Corrêa *et al*., 2019). Gram-negative bacteria exhibit greater resistance to polyphenols than their Gram-positive counterparts due to differences in their cell wall compositions (Piekarska-

Radzik & Klewicka, 2021). One potential mechanism of polyphenol action on bacterial cells is their ability to bind to bacterial cell membranes in a dose-dependent manner (Cardona *et al*., 2013). Overall, TDB24, identified as *A. nosocomialis* strain AE6 from feces, produces the most tannase equivalent of gallic acid on day 4, reaching the concentration of 42.08 U/mL. This ability could be attributed to its defense mechanisms or nutrient utilization, enabling it to withstand and break down tannins (Bhardwaj *et al*., 2003). Thus, it could be inferred that these bacteria evolved in the GIT, enabling them to degrade tannin, potentially for use in feed supplements or direct-fed microbial (DFM) to overcome forage toxicity and improve rumen fermentation (Gheibipour *et al*., 2022).

The investigation acknowledges existing research gaps in understanding TDB, emphasizing the need for further exploration, including characteristic analysis and antimicrobial profiles. Further research on tannase production in *A. nosocomialis* and exploring its characteristics can bridge existing knowledge gaps. Hence, this study successfully characterizes the identified TDB, emphasizing A*. nosocomialis* as an effective microbe in tannin degradation.

CONCLUSION

This study successfully identified ten tannin-degrading bacteria (TDB) through DNA sequencing: *B. cereus* (2), *L. macrolides* (1), *A. nosocomialis* (6), and *S. saprophyticus* (1). Among them, three isolates (TDB24, 18, and 17) exhibited high tolerance to tannin of up to 1%. The tannase content was determined through a five-day assay, with *A. nosocomialis* strain AE6 (TDB24) identified as the most efficient producer, attaining a tannase production of 42.08 U/mL on the fourth day of incubation. Future research should prioritize investigating the protein-degrading properties of identified TDBs to enhance their applicability in animal nutrition and welfare in general. Furthermore, attention should be given to optimizing the fermentation conditions of these bacteria, with a thorough understanding of factors such as pH, temperature, and nutrient concentrations. These endeavors have the potential to introduce innovative applications for tannin-degrading bacteria, contributing significantly to advancements in sustainable agriculture and animal nutrition.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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