

Growth and hematological effects of cellulolytic *Bacillus* spp. isolated from rabbit gut

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Abstract. Turaeva B, Kutlieva GJ, Kamolova XF, Nematova MA. 2026. Growth and hematological effects of cellulolytic *Bacillus* spp. isolated from rabbit gut. *Biodiversitas* 27 (1): d270131. <https://doi.org/10.13057/biodiv/d270131>. This study aimed to isolate cellulolytic *Bacillus* strains from the rabbit gastrointestinal tract and evaluate their probiotic potential. Thirteen spore-forming isolates were obtained, of which *Bacillus subtilis* PBUZ-1 and PBUZ-3 exhibited the strongest cellulolytic activity. Both strains demonstrated high cellulase activity, with PBUZ-1 producing 1.69±0.16 U/mL in whey-based medium compared to 0.29±0.02 U/mL in Getchenson medium ($p<0.001$, Cohen's $d = 12.49$). In vivo trials were conducted on Hikol rabbits supplemented with lyophilized strains (1×10^6 CFU/g feed) for 30 days. Rabbits receiving probiotics showed superior growth performance, with body weights exceeding controls by 312-1050 g at 110-120 days ($p<0.01$, Cohen's $d = 2.45-3.83$). Hematological analysis revealed significant increases in red and white blood cell counts, hemoglobin, and platelets ($p<0.05$). Biochemical profiles also improved, with higher total protein (65.17±2.24 g/L) and albumin (4.7±0.30 g/L), while bilirubin remained within physiological ranges. No adverse effects were observed, and physiological parameters such as temperature and heart rate stayed within normal limits. These findings demonstrate that rabbit gut-derived *Bacillus subtilis* strains enhance growth, hematological indices, and systemic health in rabbits. Importantly, this study provides the first evidence that such strains possess dual cellulolytic and probiotic functions, offering a safe and sustainable alternative to synthetic feed additives in rabbit farming and potentially other herbivorous livestock.

Keywords: *Bacillus subtilis*, cellulolytic bacteria, gut microbiota, probiotics, rabbit physiology

INTRODUCTION

Cellulose is one of the most abundant organic polymers in nature and constitutes the primary structural component of plant cell walls. Despite its abundance, cellulose is highly resistant to degradation due to its crystalline structure and water insolubility. Microorganisms play a central role in cellulose breakdown by producing synergistic enzymes like endoglucanases, exoglucanases, and β -glucosidases that convert cellulose into glucose, a readily utilizable energy source (Dicks et al. 2015; Thomas et al. 2018; Nematova and Murodova 2024).

Evidence indicates that native *Bacillus* strains can enhance nutrient absorption and growth by improving fiber breakdown. The *Bacillus subtilis* strains from herbivore intestines have demonstrated enhanced cellulase activity in Carboxy-Methyl Cellulose (CMC)-based media (Wita et al. 2019). Such strains also exhibit tolerance to various environmental conditions, including pH and temperature shifts, which further supports their suitability as probiotics in livestock systems (Mancini and Paci 2021; Liu et al. 2022).

Cellulolytic microorganisms are increasingly utilized in biotechnology sectors such as bioenergy, agriculture, textiles, and waste management (Abdel-Aziz et al. 2021). Among them, *Bacillus* spp. are widely favored due to their resilience, spore-forming ability, and efficient secretion of

extracellular cellulases, making them ideal for scalable enzyme production (Guo et al. 2017). Recent work has shown that cellulolytic *Bacillus cereus* strains not only degrade fiber efficiently but also produce short-chain fatty acids with probiotic potential (Liao et al. 2024).

The gastrointestinal tract of herbivores, including rabbits (*Oryctolagus cuniculus* (Linnaeus, 1758)), harbors complex microbial communities that facilitate fiber digestion and support host health (Manjunatha et al. 2016). Since rabbits rely heavily on their gut microbiota to degrade fibrous feed, isolating cellulolytic bacteria from their own digestive system could yield probiotic strains better adapted to the host environment (Ivannikova et al. 2021). Unlike soil- or feces-derived strains, host-specific isolates may provide superior colonization and physiological compatibility. Evidence suggests that host-derived probiotics can exert stronger effects compared to commercial preparations due to their natural adaptation to the gastrointestinal milieu (Fu et al. 2024).

Despite growing interest in *Bacillus*-based probiotics for animal nutrition, limited research has focused on the cellulolytic potential of strains specifically isolated from the rabbit gut. Most available probiotics are either soil-derived or commercially formulated, with uncertain adaptation to the rabbit's unique gut environment. This gap restricts the development of effective, host-adapted probiotic feed formulations.

Cellulose degradation is not only critical for nutrient cycling and digestion but also for reducing agricultural waste. Identifying strains that exhibit strong cellulase activity across a range of physicochemical conditions especially in acidic environments typical of herbivore stomachs is essential for probiotic development. Additionally, successful colonization and beneficial interaction with the host microbiota are vital for consistent probiotic performance (Ghiasi et al. 2024)

Our preliminary work yielded several spore-forming, Gram-positive *Bacillus* strains from rabbit stomach and gastric juice samples. Morphological characterization and molecular identification through 16S rRNA gene sequencing and MALDI-TOF MS confirmed two high-performing isolates as *B. subtilis* PBUZ-1 and PBUZ-3. These strains exhibited strong hydrolytic activity and grew optimally in whey-based media at pH 5.5, suggesting functional adaptation to the host gastrointestinal tract.

Subsequent in vivo trials demonstrated improved growth rates and hematological health in rabbits supplemented with lyophilized forms of these strains. These findings align with earlier reports of probiotic *Bacillus* strains enhancing feed utilization and immunity in livestock (Wang et al. 2020). Given the increasing demand for sustainable, antibiotic-free animal production systems, indigenous cellulolytic bacteria offer a promising alternative to synthetic feed additives. Host-derived strains are particularly valuable for their natural compatibility, safety, and probiotic efficacy. Therefore, this study aimed to isolate cellulolytic *Bacillus* strains from the rabbit gastrointestinal tract and evaluate their probiotic potential.

We hypothesize that cellulolytic *Bacillus* strains isolated from the rabbit gut can enhance growth performance, blood parameters, and overall health in rabbits, thereby supporting their application as host-specific probiotics in rabbit farming.

MATERIALS AND METHODS

Experimental design

The study was conducted in 2024 at the Institute of Microbiology, Academy of Sciences of Uzbekistan (Tashkent). A total of 20 healthy adult rabbits (Hikol breed) were randomly divided into four groups (n = 5 per group). Stomach and intestinal contents were aseptically collected from each animal for microbiological analysis. All animal procedures were approved by the Institutional Animal Ethics Committee (Approval No. IMAS-AEC-2024-11) and conducted according to ethical guidelines.

Sample collection and bacterial isolation

The stomach was specifically targeted rather than the cecum, since our focus was on identifying cellulolytic bacteria that can tolerate and function in the acidic gastric environment, which is critical for probiotic adaptation during passage through the upper gastrointestinal tract. Gastric juice was collected individually from each rabbit and not pooled, in order to preserve strain-level diversity. Samples were diluted in sterile saline, serially diluted, and plated on Carboxy-Methyl Cellulose (CMC) agar. After

aerobic incubation at 37°C for 48 h, colonies showing clear hydrolysis zones following iodine staining were selected as cellulolytic isolates.

Screening and identification of cellulolytic isolates

Selected colonies were cultured in CMC broth and assessed for cellulase activity using the Somogyi-Nelson method. Morphological identification included Gram staining, motility, and spore formation. Molecular identification was conducted using 16S rRNA gene sequencing and MALDI-TOF MS.

Cellulase activity assay

Quantitative cellulase activity was measured in broth containing CMC or wheat bran as the carbon source. Reducing sugars released were quantified using the Somogyi-Nelson method with slight modifications, where glucose was used as the standard and absorbance was read at 610 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of glucose per minute under assay conditions.

Optimization of growth conditions

To determine optimal enzyme production, isolates were cultured under different pH (5.5, 6.0, 6.8) and temperature (10°C, 37°C, 45°C) conditions using three media: Getchenson broth, meat peptone broth, and a specially formulated whey-based production medium (SP). The SP medium contained: whey (1 L), sodium citrate (6 g), peptone (10 g), manganese sulfate (MnSO₄, 0.018 g), magnesium sulfate (MgSO₄, 0.1 g), sodium acetate (1 g), yeast extract (0.5 g), glucose (10 g), lactose (5 g), and ammonium sulfate ((NH₄)₂SO₄, 0.3 g). The final pH was adjusted to 6.5. Colony-forming units and cellulase activity were recorded after 48 hours of incubation.

In vivo experiments

Rabbits in the treatment groups received feed supplemented with lyophilized *B. subtilis* PBUZ-1, PBUZ-3, or a combination of both for 30 days. The probiotic dosage was standardized at 1 \times 10⁶ CFU/g of feed and administered once daily. The control group was fed basal diet without supplementation. Clinical signs, including activity, fur condition, and appetite, were monitored daily, and clinical assessments were conducted under blinded conditions to minimize bias. Blood samples were collected from the marginal ear vein on days 0 and 30 for hematological and biochemical analysis. Parameters measured included hemoglobin, red blood cell count, total protein, albumin, and bilirubin.

Statistical analysis

One-Way ANOVA was used to evaluate the effect of pH, temperature, and media on growth and enzyme production. Student's t-test was applied to assess differences between control and treated groups. Statistical significance was set at p<0.05. Data are presented as mean \pm standard deviation (n = 5).

RESULTS AND DISCUSSION

Isolation

A total of 13 spore-forming bacterial isolates were obtained from pooled stomach and gastric juice samples of rabbits, collected aseptically to reduce contamination and enrich for aerobic, endospore-forming bacteria, particularly *Bacillus* spp. Although fiber fermentation primarily occurs in the cecum, the stomach was targeted to isolate *Bacillus* species more likely to colonize the upper gastrointestinal tract and exhibit strong enzymatic activity.

The isolates exhibited distinct colony morphologies and were preliminarily identified via Gram staining and light microscopy. They appeared as Gram-positive, rod-shaped cells (0.5–0.9 μm wide, 2–6 μm long), some forming short chains. Oval endospores were clearly visible under 400 \times magnification using XSP-136 B and OLYMPUS BX41 microscopes, confirming their resilience.

All isolates were cultured in sterile MRS and nutrient agar (HiMedia, India) at 37°C for 3 days at a spore concentration of 10⁸ spores/mL. Based on morphological features, they were identified as aerobic or facultatively anaerobic *Bacillus* spp. (Figure 1).

Further identification was carried out using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). All isolates showed score values >2.0, confirming reliable species-level identification. These findings were consistent with morphological and biochemical traits. The resilience of *Bacillus* spores to environmental stressors, such as desiccation, UV radiation, and oxidizing agents, underscores their suitability as candidates for probiotic application.

Cellulase activity

Among the 13 isolates, PBUZ-1 and PBUZ-3 exhibited the highest cellulolytic activity, forming clear hydrolysis zones on CMC agar with mean diameters of 23.6 \pm 1.2 mm and 28.4 \pm 1.4 mm, respectively. Both strains also showed strong degradation zones on wheat bran-based media, with PBUZ-3 being the most active (Figure 4.A). Quantitative assessment using the Somogyi-Nelson method revealed peak enzyme activities in isolate 1 (*Bacillus* sp., 0.233 U/mL) from intestinal juice and isolate 4 (*B. subtilis* sp., 0.193 U/mL) from the stomach (Figure 2). These two isolates were selected for further molecular identification and in vivo experiments based on their statistically validated cellulolytic potential.

Molecular identification and phylogeny

The cellulolytic strains PBUZ-1 and PBUZ-3 were subjected to molecular identification via 16S rRNA gene sequencing. Approximately 1.5 kb gene fragments were amplified and analyzed using the NCBI BLAST algorithm. The sequences showed 99.8–100% similarity to *Bacillus subtilis*, confirming their classification at the species level.

Phylogenetic analysis was conducted using MEGA 11 software, applying the neighbor-joining method with 1,000 bootstrap replicates. The resulting tree positioned both

PBUZ-1 and PBUZ-3 within a robust clade of *B. subtilis* (Figure 3).

The congruence between molecular (16S rRNA) and proteomic (MALDI-TOF MS) methods supports the reliability of taxonomic assignment and validates their selection for further functional studies.



Figure 1. Isolation of pure bacterial isolates from rabbit. A. Gastric juice and B. Stomach

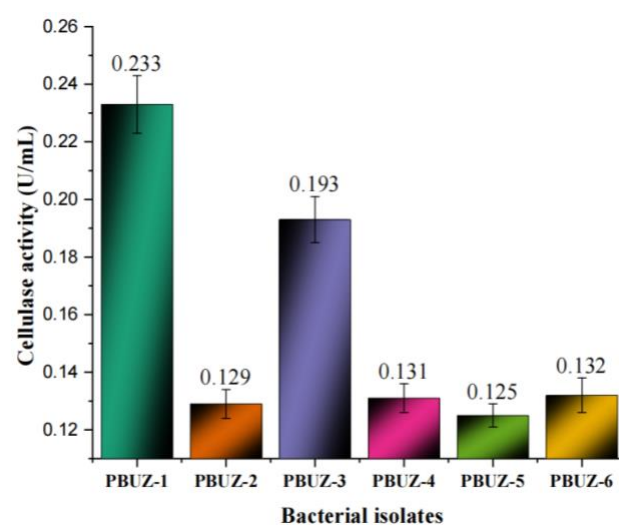


Figure 2. Cellulase activity of different bacterial isolates (n = 5, p<0.05)

Optimization of growth conditions

To enhance cellulolytic performance, the effects of pH, temperature, and culture media on the growth of *B. subtilis* PBUZ-1 and PBUZ-3 were evaluated. The strains were cultivated at pH 5.5, 6.0, and 6.8; temperatures of 10°C, 37°C, and 45°C; and in three media: Getchenson medium, meat peptone broth, and a specially formulated production medium derived from dairy by-products.

Both strains achieved CFU counts of 10^6 - 10^7 /mL under all conditions, with the highest counts and cellulase activity observed in the custom medium at pH 5.5 after 48 hours of incubation (Figure 4.B). Data are presented as mean \pm SD (n = 5). Significant differences between media were determined using ANOVA (*p<0.01, **p<0.001). Effect sizes (Cohen's d) are shown to indicate biological relevance.

Quantitative enzyme analysis revealed that all bacterial isolates exhibited significantly higher cellulase activity in SP medium compared to Getchenson. Specifically, PBUZ-1 showed 1.69 ± 0.16 U/mL in SP versus 0.29 ± 0.02 U/mL in Getchenson (*p<0.001, Cohen's d = 12.49). PBUZ-2 exhibited 1.30 ± 0.11 U/mL in SP. These results demonstrate the superior suitability of SP medium for cellulase production. The very large effect size observed for PBUZ-1 confirms the biological significance of the medium,

supporting its use for optimized enzyme production and subsequent lyophilization for in vivo applications.

Growth effects

A 30-day in vivo trial was conducted on healthy Hikol rabbits divided into four groups: PBUZ-1, PBUZ-3, a combination of both strains, and a non-treated control. Lyophilized bacterial strains were incorporated into the feed of the treatment groups, while the control group received the same diet without any supplementation.

Rabbits receiving probiotics exhibited improved clinical signs, including increased activity, better feed intake, and healthy fur condition. No signs of distress or mortality were observed during the experimental period. These improvements were evident externally - rabbits appeared more energetic, had better appetites, and showed glossier coats. Live weights were recorded at 90, 110, and 120 days of age. Starting from day 20, rabbits in the experimental groups receiving *Bacillus*-derived probiotics exhibited faster growth. By day 110, body weight in the experimental groups exceeded that of the control by 312 g, 555 g, and up to 1050 g. Probiotic supplementation enhanced growth performance, resulting in higher body weight gain and improved physical development in experimental rabbits (Table 1).

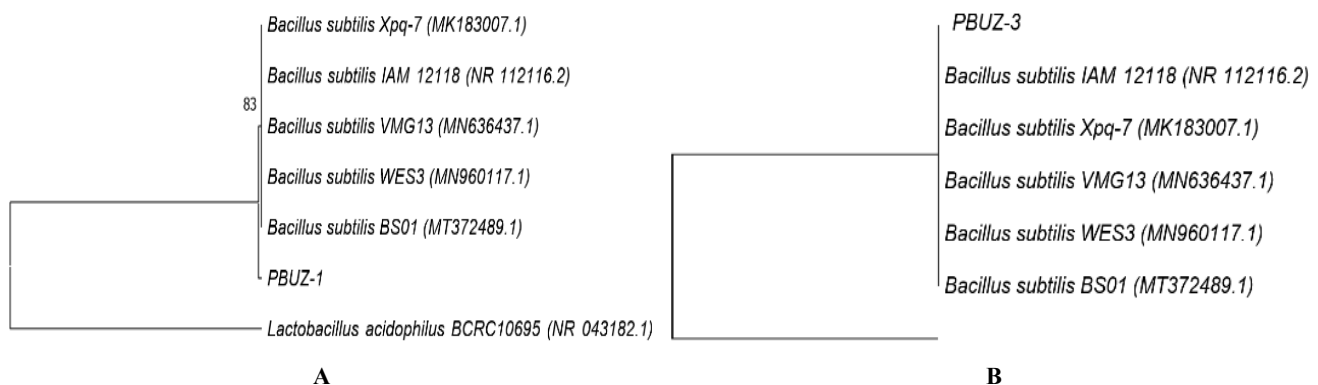


Figure 3. Position of: A. *Bacillus subtilis* PBUZ-1, and B. *Bacillus subtilis* PBUZ-3 strains in the phylogenetic tree

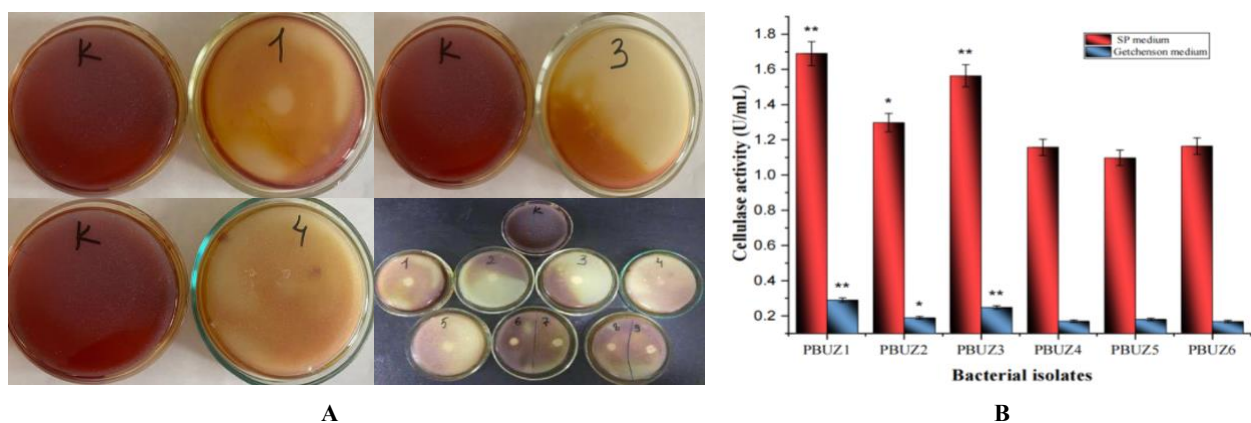


Figure 4. A. Primary screening of bacterial strains for cellulolytic activity, B. Cellulase activity of bacterial isolates grown in SP and Getchenson media

Physiological parameters

To optimize dosage, two concentrations of lyophilized cellulolytic *Bacillus* strains were tested: 2 g/kg feed (Experiment 1) and 4 g/kg feed (Experiment 2). Controls received the same diet without supplementation. Physiological parameters such as body temperature, heart rate, and respiration rate were monitored to evaluate overall health (Table 2). Physiological parameters including body temperature, heart rate, and respiratory rate were recorded before and after the experiment. Post-experimental measurements showed slight but significant reductions in body temperature and heart rate in Experimental-1 (* $p < 0.05$) and Experimental-2 (** $p < 0.01$) compared to controls. Respiratory rate decreased slightly in Experimental-2 (* $p < 0.05$). All values remained within the normal physiological range.

Hematology

Hematological assessments on day 30 showed significant increases in red and white blood cell counts, hemoglobin levels, and platelet numbers across all treated groups,

particularly in the PBUZ-3 and combined groups ($p < 0.05$ vs. control). Baseline measurements on day 0 confirmed no initial differences among the groups, indicating physiological comparability at the start of the experiment. Additionally, treated animals exhibited elevated basal phagocytic activity (PhP) and enhanced assimilation processes, suggesting potential immunomodulatory effects of the bacterial strains.

Furthermore, the effects of supplementation on serum albumin, bilirubin, and total protein levels were evaluated, with results summarized in Table 3. Serum albumin and total protein levels were higher in Experimental-1 and Experimental-2 compared to controls, while bilirubin was elevated in the control group above the normal reference range. Total protein in the control group fell below the physiological minimum. These findings suggest that probiotic supplementation supported normal protein metabolism, whereas deviations in the control group may indicate potential liver dysfunction or reduced protein synthesis.

Table 1. Growth performance of rabbits in control and experimental groups at different ages

Selected rabbits for the experimental groups	Live weight of rabbits (g)		
	(90 days old)	(110 days old)	(120 days old)
Control	2855±110	3118±125	3350±140
Experimental	2763±115	3430±130*	3905±150**

Note: Data are presented as mean±SD, $n = 5$. * $p < 0.05$, ** $p < 0.01$ vs. control (ANOVA with post hoc test). Cohen's $d = 2.45$ for 110 days, $d = 3.83$ for 120 days, indicating the magnitude of effect

Table 2. Effect of cellulolytic *Bacillus* strains on the physiological parameters of rabbits

Parameter	Normal range	Pre-experiment	Post-experimental status		
			Experimental-1	Experimental-2	Control
Body temperature (°C)	38.5-39.5	37.6±0.01	38.1±0.10 *	38.2±0.04 *	39.02±0.31
Heart rate (beats/min)	120-160	146±1.7	143.8±1.3 *	141±1.2 **	149.6±1.4
Respiratory rate (/min)	50-60	54.2±0.7	52.6±0.7	52.8±0.5 *	54.4±0.4

Note: Values are presented as mean±SD ($n = 5$). * $p < 0.05$, ** $p < 0.01$ vs control (ANOVA with post hoc test). Cohen's d indicates effect size: Body temperature: Exp-1 = -3.99, Exp-2 = -3.71, Heart rate: Exp-1 = -4.29, Exp-2 = -6.60, Respiratory rate: Exp-1 = -3.16, Exp-2 = -3.53

Table 3. Effect of cellulolytic *Bacillus* strains on biochemical parameters of rabbit blood

Biochemical parameters	Normal range	Groups		
		Experimental-1	Experimental-2	Control
Albumin g/L	3.0-5.0	4.3±0.30	4.7±0.30	5.6±0.45 *
Bilirubin, $\mu\text{mol/L}$	5-13	7.3±1.18	8.29±0.88	14.06±0.62
Total protein g/L	59-74	61.7±3.28	65.17±2.24	51.14±1.91 **

Note: Values are presented as mean±SD ($n = 5$). * $p < 0.05$, ** $p < 0.01$ vs control (ANOVA with post hoc test). Cohen's d (effect size vs control): Albumin: Exp-1, $d = -3.04$, Exp-2, $d = -2.00$, Bilirubin: Exp-1, $d = -5.77$; Exp-2, $d = -8.40$, Total protein: Exp-1, $d = 3.41$, Exp-2, $d = 7.22$

Discussion

The findings of this study reinforce previous evidence supporting the beneficial role of *Bacillus* species in rabbit nutrition. The observed improvements in body weight, erythrocyte count, hemoglobin levels, and feed efficiency are consistent with studies on *B. subtilis* and related strains (Dobrzyński et al. 2023). These results suggest that host-derived probiotic strains can successfully colonize the gastrointestinal tract and enhance physiological functions.

Comparable outcomes have been documented in trials involving *B. velezensis* and *B. amyloliquefaciens*, which demonstrated strong enzymatic activity and contributed to improved gut health in herbivorous animals (Ngalimat et al. 2021; Borah et al. 2025; Chen et al. 2025; Khongkool et al. 2025). Notably, while these studies largely emphasized gut microbial modulation and digestive efficiency, our work extends this knowledge by highlighting improvements in hematological indices—an aspect rarely addressed in herbivore trials. This indicates that cellulolytic *Bacillus* spp. may not only optimize digestion but also enhance systemic metabolism and oxygen transport.

Mechanistically, the positive effects observed in rabbits may be attributed to cellulolytic enzyme production and Short-Chain Fatty Acid (SCFA) synthesis, which enhance fiber digestion and provide additional energy sources (Sun et al. 2022; Liao et al. 2024; Pu et al. 2025). Previous poultry studies linked *Bacillus*-driven growth improvements mainly to gut flora modulation, whereas our results suggest that enzymatic fiber degradation and SCFA production play a more central role in lagomorphs. This highlights a potential host-specific mechanism that differentiates rabbit responses from those of other non-ruminants.

Our findings also support earlier reports indicating that *Bacillus* probiotics can improve hematological parameters, such as red blood cell count and hemoglobin concentration, as demonstrated in rabbits by Abdelsalam et al. (2025). However, unlike Abdelsalam's study, which used a commercial preparation in rabbits, our study introduces the novel concept that rabbit gut-derived strains can produce similar systemic benefits, thereby broadening the host range for probiotic application.

Additionally, the current findings align with recent investigations into gut-associated *Bacillus* spp. in non-ruminant hosts. For instance, *B. subtilis* isolated from ruminants showed significant endoglucanase activity and promoted growth in pigs and poultry (Mun et al. 2021). Similarly, *B. velezensis* from pig intestines enhanced feed conversion rates through cellulase secretion (Cai et al. 2024). In rabbits, *B. subtilis* strains have been identified with potent enzyme production and immune-stimulating properties (Zheng et al. 2020). While these studies underscore the broad potential of *Bacillus* probiotics, our results specifically demonstrate the advantages of host-adapted strains, which appear to tolerate diverse environmental conditions and more effectively support systemic health compared to non-host-derived strains.

Nevertheless, several limitations should be acknowledged. First, bacterial isolation was conducted only from stomach samples, excluding the cecum, the primary site of fiber fermentation in rabbits. This contrasts

with earlier studies, where cecal isolates provided broader insight into cellulolytic diversity. Second, the in vivo trial involved a relatively small number of animals ($n = 5$ per group), which may limit the statistical power of the results compared to larger-scale animal studies. Third, although taxonomic identification was performed via 16S rRNA gene sequencing, the study did not include gut microbiota profiling, thereby restricting broader insights into microbial community interactions.

Future studies should address these limitations by including cecal sampling, larger animal cohorts with increased replication, and advanced techniques such as metagenomics or high-throughput sequencing to better understand the interactions between probiotics and host microbiota. Additionally, long-term field trials are recommended to validate the efficacy of these strains under practical farming conditions and to evaluate their economic feasibility.

Overall, this study demonstrates that rabbit gut-derived cellulolytic *Bacillus* probiotics improve digestion, stimulate growth, and enhance both systemic and hematological health. These effects likely occur via synergistic interactions with the native gut microbiota, SCFA production, and cellulolytic enzyme activity. Compared to previous research, our findings highlight a novel, host-specific probiotic approach in rabbits and set the stage for future functional genomic studies to optimize probiotic formulations for lagomorph nutrition.

In conclusion, this study provides the first evidence that rabbit gut-derived cellulolytic *Bacillus subtilis* strains can simultaneously enhance growth performance and hematological parameters, thereby improving both digestive efficiency and systemic health in lagomorphs. By producing cellulases and promoting fiber degradation, these host-adapted strains reduce reliance on costly synthetic enzymes, offering a natural and sustainable alternative for rabbit farming. Importantly, no adverse effects were observed, highlighting their biosafety and compatibility with the host. Their dual role as enzyme producers and probiotics underscores their potential as innovative feed additives not only for rabbits but also for other herbivorous livestock.

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