Postprint: Isolation, Identification and Biological Characterization of Pig-derived Probiotic Bacillus

Authors: Jiang Jiaxuan, Wang Shujing, Ren Zhihong, Du Huamao

Date: 2017-10-11T00:00:00+00:00

Abstract

This study aimed to screen Bacillus strains with favorable characteristics from the normal intestinal flora of healthy pigs, for the purpose of improving feed utilization efficiency, reducing porcine gastrointestinal tract infections, and decreasing post-infection antibiotic usage. Multiparous sows with excellent production performance were selected from remote mountainous regions of Chongqing that had never been fed compound feed. Acid- and bile salt-resistant Bacillus strains were isolated from their fresh feces, and further screened for the ability to simultaneously produce amylase, cellulase, and protease. Finally, biochemical identification and molecular identification based on 16S rDNA sequencing were performed. The results demonstrated that a total of 7 Bacillus strains capable of simultaneously secreting amylase, cellulase, and protease were obtained. These strains exhibited survival rates of 30%–90% after treatment at pH 3.0 for 2 h, and 40%–100% after treatment in 0.5% bile salts for 2 h. Biochemical and molecular identification revealed 1 strain of Bacillus stearothermophilus, 1 strain of Bacillus licheniformis, and 5 strains of Bacillus subtilis. These findings indicate that the 7 Bacillus strains screened in this study can tolerate low-pH and high-bile-salt environments, and possess the capability to simultaneously secrete three extracellular enzymes: amylase, cellulase, and protease.

Full Text

Isolation, Identification, and Biological Characterization of Probiotic Bacillus spp. from Swine

JIANG Jiaxuan[1], WANG Shujing[1], REN Zhihong[1], DU Huamao[2]*

[1]College of Biotechnology, Southwest University, Chongqing 400715, China
[2]State Key Laboratory for Infectious Disease Prevention and Control, National

Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

Abstract: This study aimed to screen Bacillus strains with robust performance from the normal intestinal flora of healthy pigs for applications in improving feed utilization, reducing digestive tract infections, and decreasing post-infection antibiotic use. We selected multiparous sows with excellent production performance from remote mountainous areas of Chongqing that had never been fed formulated diets, and isolated acid- and bile salt-tolerant Bacillus strains from their fresh feces. Strains capable of simultaneously producing amylase, cellulase, and protease were further screened and subsequently identified through biochemical assays and molecular analysis based on 16S rDNA sequencing. The results demonstrated that seven Bacillus isolates could secrete all three enzymes, exhibiting survival rates of 30%–90% after 2-hour treatment at pH 3.0 and 40%–100% after 2-hour exposure to 0.5% bile salts. Biochemical and molecular identification revealed one strain of *Bacillus stearothermophilus*, one strain of *Bacillus licheniformis*, and five strains of *Bacillus subtilis*. These findings indicate that the seven selected Bacillus strains possess strong tolerance to low pH and high bile salt conditions while maintaining the capacity to simultaneously secrete three extracellular enzymes: amylase, cellulase, and protease.

Keywords: probiotics; *Bacillus* spp.; biochemical identification; 16S rDNA; enzyme production capacity

---

The rampant abuse of antibiotics has led to the emergence of drug-resistant and super-resistant bacterial strains that pose severe threats to human health, making the development of microbial feed additives a critical direction for antibiotic replacement in animal production. China approved 34 directly feed-additive microorganisms in 2013, while the U.S. Food and Drug Administration (FDA) approved 42, among which six belong to the *Bacillus* genus [**?**]. In addition to inhibiting pathogenic bacteria through competitive exclusion and oxygen depletion, *Bacillus* probiotics enhance feed utilization and promote livestock growth by producing various hydrolytic enzymes and growth-stimulating factors in the intestinal tract. Ye et al. [**?**] measured D/d values (transparent zone diameter to colony diameter ratio) of 2–4 for three enzymes produced by swine-derived *Bacillus* strains using the plate assay method. Tang et al. [**?**] and Xie et al. [**?**] isolated *Bacillus* strains from soil to evaluate amylase activity and employed mutagenesis breeding to double the D/d values. However, most reported studies have focused on strains producing only one or two types of enzymes, while *Bacillus* capable of simultaneously secreting multiple extracellular enzymes remains underexplored. Strains that can concurrently produce amylase, cellulase, and protease offer greater nutritional benefits. This study aimed to isolate *Bacillus* strains from the feces of multiparous sows with excellent production performance raised by farmers in remote mountainous regions without formulated feed supplementation, selecting for isolates with strong tolerance to low pH and high bile salts that could simultaneously produce all three enzymes, thereby establishing

a foundation for developing species-specific probiotic formulations for swine feed applications.

Materials and Methods

1.1 Experimental Materials

Fresh fecal samples were collected from Taihu sows raised by individual farmers in Beibei, Chongqing. The reference strain S1-2 was obtained from a selenium-enriched *Bacillus subtilis* feed additive (viable count 5$×10^{10}$ CFU/g, Shenwei Microbial Strain Technology Co., Ltd., GB/125884–2010). Culture media included LB medium, starch medium, casein medium, and sodium carboxymethyl cellulose medium, along with ox bile salts and hydrochloric acid. Biochemical identification kits (API50CHB/E and API50CH) were purchased from Beijing Weitaike Biotechnology Co., Ltd., and bacterial genomic DNA extraction kits from Beijing Tsingke Xinye Biotechnology Co., Ltd.

1.2 Experimental Methods

1.2.1 Initial Screening of Bacillus Strains Small amounts of frozen fecal specimens were thawed, vortexed, and aliquoted. One portion was heat-treated at 80°C for 15 minutes, serially diluted, and plated onto LB agar (50 L per plate). After incubation at 37°C for 18–24 hours, distinct single colonies were selected and purified through two successive generations.

1.2.2 Acid and Bile Salt Tolerance Tests Vigorous single colonies were inoculated into 1 mL LB broth and cultured at 37°C with shaking (120 rpm) for 6 hours. The cultures were then transferred (5% inoculum) to phosphate-buffered saline (PBS) at pH 3.5, with pH 7.0 PBS serving as the control. After 2 hours, serial dilutions were plated in duplicate for each dilution factor. Following 18-hour incubation at 37°C, viable colony counts were performed and survival rates were calculated using the formula: Survival rate (%) = (colony count in test group / colony count in control group) × 100. Strains showing >40% survival in the acid tolerance test were selected for bile salt tolerance evaluation. Six-hour cultures were transferred (5% inoculum) to LB medium containing 0.5% ox bile salts, with bile-free LB as the control. After 24-hour incubation, cultures were serially diluted and plated in duplicate. Plates were incubated for 18 hours before viable counting and survival rate calculation.

1.2.3 Enzyme Production Assays

1.2.3.1 Amylase Production Assay Following the method described in reference [**?**], single colonies were picked with sterile toothpicks and spot-inoculated onto starch plates in triplicate, with one water control spot per plate. After 24-hour incubation at 37°C, 1 mL of iodine solution was added to uniformly cover the plate, which was then kept at 4°C for 10 minutes. The transparent zone

diameter (D) and colony diameter (d) were measured, and the D/d ratio was calculated and averaged across replicates.

1.2.3.2 Cellulase Production Assay Following reference [**?**], test strains were spot-inoculated onto sodium carboxymethyl cellulose plates in triplicate with one water control. After 24-hour incubation at 37°C, plates were stained with 0.2% Congo red for 30 minutes, then destained sequentially with distilled water and 1 mol/L NaCl for 5 minutes each, followed by color fixation with 5% acetic acid for 5 minutes. D and d values were measured and D/d ratios calculated and averaged.

1.2.3.3 Protease Production Assay Following reference [**?**], strains were spot-inoculated onto casein plates in triplicate with one water control and incubated at 37°C for 48 hours. D and d measurements were taken and D/d ratios calculated and averaged.

1.2.4 Enhanced Acid and Bile Salt Tolerance of Isolates To select strains more suitable for commercial applications, those capable of producing all three enzymes were subjected to further acid tolerance analysis by measuring survival rates after 2-hour exposure to pH 2.0, 2.5, and 3.0. Bile salt tolerance at 0.5% concentration was also re-evaluated.

1.2.5 Biochemical Identification Strains with D/d values >1 and both D and d >2 mm in all three enzyme assays were selected for microbiological identification. After 18-hour culture on LB plates, several consistent colonies were suspended in API50CHB/E medium to achieve a turbidity equivalent to 2 McFarland standard. One drop of suspension was added to each reaction well of the API50CH strip, overlaid with mineral oil, and incubated at 37°C. Biochemical reaction results were recorded at 24 and 48 hours, and identification was performed according to the API bacterial identification system manual.

1.2.6 16S rDNA Sequence Analysis Genomic DNA was extracted from each strain using the bacterial genomic DNA extraction kit according to the Gram-positive bacterial protocol. The 16S rDNA was amplified by PCR using primers 27F (5' -AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' -TACGGCTACCTTGTTACGACTT-3') as described in reference [**?**]. PCR products were sent to Tsingke Company (Beijing) for bidirectional sequencing. Sequences were subjected to BLAST analysis in the NCBI database to identify strains with >99% similarity, and phylogenetic trees were constructed using MEGA 6.0 software with the neighbor-joining method.

1.2.7 Statistical Analysis SPSS 19.0 software was used for statistical analysis of D/d values. Multiple comparisons were performed using the LSD method, and data are presented as mean ± standard deviation. $P<0.05$ and $P<0.01$ were considered statistically significant and highly significant, respectively.

Results and Analysis

Nine fecal samples were heat-treated at 80°C for 15 minutes and plated, yielding 52 isolates after 24-hour incubation. These isolates and the reference strain S1-2 were tested for acid tolerance (pH 3.5, 2 hours) and bile salt tolerance (0.5% bile salts, 24 hours), resulting in 13 strains with survival rates >40% in both tests that were selected for enzyme production screening.

2.1 Enzyme Production Capacity

The clear zone method was used to qualitatively detect amylase, protease, and cellulase production, where transparent halos indicate enzyme secretion. The D/d ratio reflects enzyme production capacity; values >1 indicate extracellular enzyme secretion, with higher values representing stronger production. The commercial reference strain S1-2 and seven isolates (P1.1-4, P1.1-10, P1.2-1, P1.2-3, P1.3-1, P1.3-5, P1.4-2) simultaneously produced all three enzymes (Figure 1 [Figure 1: see original paper]). Variation in halo sizes among strains suggested differences in enzyme production capabilities. Six other strains that did not express all three enzymes were excluded from further analysis, though notably, strain S1.1-10, despite lacking amylase activity, exhibited protease D/d values three times higher than S1-2.

D and d measurements were taken to calculate D/d ratios, with means and standard deviations derived from three replicate experiments and subjected to multiple comparisons (Table 1). No significant differences in cellulase production were observed between the seven isolates and S1-2 ($P>0.05$). For amylase, strains P1.1-10, P1.2-1, P1.2-3, P1.3-1, and P1.3-5 showed no significant differences from S1-2 ($P>0.05$), while P1.4-2 was significantly lower ($P<0.05$) and P1.1-4 was highly significantly higher ($P<0.01$). All isolates demonstrated strong protease production, with P1.1-10 and P1.3-1 showing highly significant increases over S1-2 ($P<0.01$), and P1.2-1 and P1.2-3 showing significant increases ($P<0.05$). No single strain exhibited high production of all three enzymes, suggesting that future probiotic formulations should combine multiple strains.

2.2 Tolerance to Acidic Conditions and Bile Salts

The pH in animal stomachs ranges from 2.0 to 4.0, with gastric emptying times of 2–4 hours. We further examined the survival rates of the seven triple-enzyme-producing strains after 2-hour exposure to pH 2.0, 2.5, and 3.0. Survival rates decreased with increasing acidity, though viable bacteria remained even under the extreme condition of pH 2.0 (data not shown). Acid tolerance varied considerably among strains, with P1.1-4 and P1.4-2 showing superior tolerance and significantly higher survival rates than S1-2 at pH 3.0. After 2-hour treatment in 0.5% bile salt medium, S1-2, P1.2-3, and P1.4-2 exhibited survival rates of 33.6%, 40.0%, and 84.4%, respectively, while the remaining five strains showed 100% survival with varying degrees of growth, including P1.1-4 and P1.2-1 which

doubled their cell numbers (Figure 2 [Figure 2: see original paper]).

2.3 Biochemical and 16S rDNA Molecular Identification

The API identification system classified P1.2-1 as *Bacillus stearothermophilus* and P1.3-5 as *Bacillus licheniformis*. Despite inconsistent fermentation reactions for glutamic acid and D-gentiobiose, the system identified P1.1-4, P1.1-10, P1.2-3, P1.3-1, P1.4-2, and S1-2 as *Bacillus subtilis* (Table 3). The API system requires an identification percentage (Id) >80% for meaningful results, with T-index values indicating strain typicality. The identification of S1-2 as *B. subtilis* was rated "excellent" (Id$ 99.9 0.75), $while P1.2-3 and P1.4-2 were "very good" (99.0 Id 99.8 $0.75), and P1.3-1 was "acceptable." Overall, all eight identifications were statistically meaningful and reliable.

16S rDNA sequencing and phylogenetic tree construction (not shown) revealed that all seven isolates showed high similarity to *Bacillus* species, though the closest phylogenetic relatives did not always match the biochemical identification results. As shown in Table 4 , S1-2, P1.1-10, P1.3-1, and P1.4-2 were most closely related to *B. subtilis*, consistent with API results, while the other three strains showed different nearest neighbors.

Discussion

3.1 Sample Source

Given the host specificity of probiotics, pig feces were selected as the isolation material for developing swine-specific feed additives. Lu et al. [**?**] reported that 454 pyrosequencing analysis of gut microbiota in piglets and fattening pigs revealed richer microbial diversity and lower pathogen proportions under natural feeding conditions compared to formulated diets. Alexopoulos et al. [**?**] demonstrated that probiotic supplements containing *Bacillus licheniformis* and *Bacillus subtilis* reduced postpartum weight loss in sows, increased feed intake and milk production, decreased mastitis-metritis-agalactia syndrome incidence, reduced piglet diarrhea, increased weight gain, and ultimately improved piglet survival rates. Regarding antimicrobial activity, Ye et al. [**?**] showed that intestinal *Bacillus* strains exhibit varying degrees of inhibition against *Staphylococcus aureus*, *Escherichia coli*, and *Shigella* species, making them prime candidates for probiotic development. Therefore, this study targeted multiparous sows from remote mountainous regions with no history of formulated feed consumption and excellent production performance, aiming to isolate probiotic *Bacillus* strains from their healthy gut microbiota.

3.2 Analysis of Acid and Bile Salt Tolerance

Lactic acid bacteria and streptococci, as indigenous gut microorganisms, have stringent nutritional requirements and growth conditions that limit industrial-scale production [**?**]. In contrast, *Bacillus* species have low nutritional demands,

simple culture conditions, and enhance feed digestibility and nutritional value while exhibiting strong resistance to harsh conditions including gastric acid, bile salts, and radiation. The stable spore form can germinate into vegetative cells in the intestine to compete with pathogens for nutrients and space while stimulating the immune system to improve host function. The seven triple-enzyme-producing strains selected in this study demonstrated survival rates of 30%–90% at pH 3.0 for 2 hours, with most showing 100% survival in 0.5% bile salts and two strains even exhibiting growth. These characteristics confirm their potential as microbial feed additives capable of competitive exclusion and oxygen depletion.

3.3 Enzyme Production Performance

All strains showed relatively low amylase and cellulase production, with D/d values around 1–2, the highest being P1.1-4 at only 2.03. Notably, four isolates demonstrated exceptionally strong protease production with D/d values of 6–7, representing 2–3-fold increases over previously reported strains [**?**]. Strain P1.1-10 exhibited the strongest protease activity with a D/d value of 10, though it lacked amylase production. However, halo size does not fully correlate with enzyme activity, meaning strains with smaller halos may still possess high enzymatic activity. The results reveal substantial physiological and biochemical diversity among *Bacillus* strains from healthy pig intestines, with significant variation in enzyme production capabilities among different strains. Therefore, microecological formulations for gut function modulation must consider enzyme production levels alongside acid and bile salt tolerance, requiring strategic combination of different strains to achieve optimal degradation of crude protein and carbohydrates, with particular emphasis on improving crude protein digestibility.

3.4 Biochemical and Molecular Identification

Two identification methods were employed for the seven selected *Bacillus* isolates: the 梅里埃 biochemical test strip with 49 reactions and 16S rDNA sequence-based molecular identification, though results were not entirely consistent. Only S1-2, P1.1-10, P1.3-1, and P1.4-2 were identified as *B. subtilis* by both methods. We provisionally adopted the API biochemical identification results pending more comprehensive molecular analysis. Discrepancies between biochemical and molecular identification are common, as 16S rDNA molecular evolution may not be fully synchronized with the evolution of housekeeping genes used for enzymatic identification, or because the compared 16S rDNA sequence length may be insufficient to represent the strain' s genetic information [**?**]. The results demonstrate clear biodiversity of intestinal *Bacillus* in healthy pigs, with *B. subtilis* as the dominant species (five isolates), plus one *B. stearothermophilus* and one *B. licheniformis*. Ning et al. [**?**] similarly isolated 13 *Bacillus* strains from pig feces, nine of which were *B. subtilis*, further supporting *B. subtilis* as the predominant *Bacillus* species in the porcine gut.

This study successfully isolated strains with good tolerance to low pH and high bile salts through heat treatment, acid tolerance, and bile salt tolerance screening. Seven superior strains capable of simultaneously secreting amylase, protease, and cellulase were selected via clear zone assays on selective media and identified as *Bacillus* species through biochemical reactions and 16S rDNA molecular analysis.

Acknowledgments: We sincerely thank Professor Xie Hefang from Southwest University for valuable guidance on statistical methods and data analysis.

References: [1] GAO Lin, BAI Zijin, FENG Bo, et al. Research and application progress of microbial feed additives[J]. Journal of Microbiology, 2014, 34(2): 1-6. [2] YE Guangbin, WANG Caihong, XIONG Li, et al. Preliminary study on enzyme-producing properties of three *Bacillus* strains[J]. Jiangsu Agricultural Sciences, 2013, 41(7): 240-242. [3] TANG Lijiang, WANG Zhenhua, WANG Di. Screening of high-yield amylase *Bacillus* strains[J]. Journal of Anhui Agricultural Sciences, 2009, 37(12): 5362-5363, 5371. [4] XIE Fengxing, ZHAO Yujie, ZHOU Ke, et al. Isolation and screening of amylase-producing *Bacillus subtilis* and its UV mutagenesis breeding[J]. Acta Agriculturae Boreali-Sinica, 2009, 24(3): 78-82. [5] ZHAO W, ZHENG J, WANG Y G, et al. A marked enhancement in production of amylase by *Bacillus amyloliquefaciens* in flask fermentation using statistical methods[J]. Journal of Central South University of Technology, 2011, 18(4): 1054-1062. [6] VIJAYARAGHAVAN P, ARUN A, AL-DHABI N A, et al. Novel *Bacillus subtilis* IND19 cell factory for the simultaneous production of carboxy methyl cellulase and protease using cow dung substrate in solid-substrate fermentation[J]. Biotechnology for Biofuels, 2016, 9: 73. [7] ASH C, FARROW J A E, WALLBANKS S, et al. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences[J]. Letters in Applied Microbiology, 1991, 13(4): 202-206. [8] LU X M, LU P Z, ZHANG H. Bacterial communities in manures of piglets and adult pigs bred with different feeds revealed by 16S rDNA 454 pyrosequencing[J]. Applied Microbiology and Biotechnology, 2014, 98(6): 2657-2665. [9] ALEXOPOULOS C, GEORGOULAKIS I E, TZIVARA A, et al. Field evaluation of the efficacy of a probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* spores, on the health status and performance of sows and their litters[J]. Journal of Animal Physiology and Animal Nutrition, 2004, 88(11/12): 381-392. [10] YE X L, LI X G, YUAN L J, et al. Relationship between the antibacterial and immunological activities of houttuyfonate homologues and their surface activities[J]. Journal of Asian Natural Products Research, 2006, 8(4): 327-334. [11] HYRONIMUS B, MARREC C L, SASSI A H, et al. Acid and bile tolerance of spore-forming lactic acid bacteria[J]. International Journal of Food Microbiology, 2000, 61(2/3): 193-197. [12] TOLBA O, EARLE J A P, MILLAR B C, et al. Speciation of *Bacillus* spp. in honey produced in Northern Ireland by employment of 16S rDNA PCR and automated DNA sequencing techniques[J]. World Journal of Microbiology and Biotechnology, 2007, 23(12): 1805-1808. [13] NING Yuchang, ZHAO Xuyong, ZHENG Ming. Isolation, identification and biological characteristics of *Bacillus* strains

from swine[J]. China Animal Husbandry and Veterinary Medicine, 2012, 39(9): 65-68.

Note: Figure translations are in progress. See original paper for figures.