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Antibiotic susceptibility profile of probiotic bacillus subtilis species

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Abstract

The study aims to determine the antibiotic tolerance patterns of Bacillus subtilis isolates extracted from local broiler chicken intestines and determine their suitability as probiotics. A study isolated Bacillus subtilis from broiler chickens' intestinal walls following organic food consumption. The identification process included morphological evaluation and biochemical tests for strain recognition, while antibiotic susceptibility testing was done through the disk diffusion method. The bacterial species were identified through PCR testing of the bsub and RecA genes, and sequence confirmation was followed by amplification. The Bacillus subtilis isolate showed susceptibility against multiple antibiotics, including trimethoprim-sulfa, kanamycin, ciprofloxacin, gentamycin, penicillin, and erythromycin. The bacterial strain demonstrated resistance to oxytetracycline, colistin sulfate, cefotaxime with clavulanic acid and ampicillin. The analysis of RecA genetic material led to the identification of Bacillus subtilis at a molecular level.

Keywords: Conventional PCR, RecA gene, rRNA gene sequences, AFLP, RAPD, Rep-PCR

Introduction

Antibiotic resistance has emerged as one of the most crucial and critical issues in today's world of the twenty-first century. The increase in the usage of antibiotics, especially in human health and in the use of feed for animals, has led to the production of multi-drug resistant bacteria, which are hard to manage, besides the risk they pose when being transferred from animals to human beings. Antibiotics have been added to animal feeds for growth promotion and prevention of diseases, and this has had ripple effects, such as the development of antibiotic resistance in animals and humans (Ahmed et al., 2024; Zhang et al., 2024). Consequently, there has been a shift to seek non-antibiotic solutions, especially in farming contexts that involve livestock. Probiotics refer to live beneficial microorganisms that have health benefits to the host upon intake in sufficient quantities and have been used in the feed industry as a safer supplement to antibiotics (Dhama et al., 2011).

In poultry farming, *Bacillus subtilis*, which belongs to the group of probiotic bacteria, is considered the most utilised species. This endospore-forming aerobic bacterium is natively associated with the gastrointestinal tract of chickens. It is well-known for enhancing positive impacts on gut health and other growth performance and the immune

system of animals. Probiotic *Bacillus subtilis* strains have the potential to inhibit the growth of pathogens such as *Clostridium*, *Streptococcus*, and *Campylobacter* and decrease the likelihood of gastrointestinal illnesses in poultry (Krysiak et al., 2021). Therefore, *Bacillus subtilis* has become popular in animal feed to promote the growth of the animals as well as to guard against diseases. *Bacillus subtilis* and most other probiotic bacteria are assumed to be safe and useful; nonetheless, their association with antibiotics – that are often used on farms – raises some questions about their ability to acquire or spread antibiotic-resistance genes.

The use of probiotics in animal feed has rapidly gained popularity, and this has led to certain concerns concerning their effectiveness, especially when used together with antibiotics. One of the bacterial species that have been found to be resistant to several of the antibiotics, as mentioned earlier, that are used in veterinary medicine is *Bacillus subtilis*, resistant to tetracyclines, ampicillin and colistin sulfate. Probiotics are widely used in animal health to help manage bacterial infections, with the said antibiotics' functionality often diminished by the existent resistant strains. Thus, probiotics are viewed as a potential substitute for antibiotics in spearing animal health. Still, the ability of these additives to maintain or transfer resistance, which is relevant to antibiotics applied in therapeutic doses, should be considered. The possibility of pathogenic bacteria acquiring antibiotic-resistance genes from probiotics is another phenomenon that deserves more research on risks in animal production systems (Arsène et al., 2021).

However, the future of these probiotics, including *Bacillus subtilis*, as a suitable substitute for antibiotics lies in the capacity of these probiotics to withstand the antibiotics that are used on animals. Strains employed in probiotics may become antibiotic-resistant, thus rebutting the effectiveness of antibiotics required to overcome infections. Hence, there is a need to assess the antibiotic susceptibility of the strains used in probiotics. This is because, with constant monitoring, deep research, and studies on the antibiotic susceptibility of these probiotics, there is a likelihood of the emergence of antibiotic-resistant strains in these products.

This study, therefore, seeks to determine the antibiotic susceptibility of Bacillus subtilis strains from local broiler chickens' intestinal tracts. By determining the susceptibility of these strains to a number of antibiotics used both in veterinary and human medicine, this study aims to highlight a gap in information on the potential dangers of using B. subtilis as a probiotic in poultry production. The relationship between probiotics and antibiotics in the context of animal production systems has become important due to the need to establish how probiotics can be used as a replacement for antibiotics in animals while preventing antibiotic resistance from increasing even further.

Materials and methods

Bacillus subtilis Isolation and Identification

Bacterial suspensions originated from the intestinal walls of broiler chickens living in the northern region that received organic food. Each sample contained mucosal material suspended in 0.9% NaCl before being subjected to boiling at 80°C for 20 minutes. The diluted bacterial solution received a 1:9 dilution step before transferring it to Nutrient Agar alongside Tryptic Soy Agar (TSA) media (Oxoid, UK) for 37°C-incubation at a 24-hour duration. The laboratory evaluated the properties of individual isolated bacterial colonies using a combination of Gram stain and oxidase, catalase, urease, starch hydrolysis, and motility tests (Cheesbrough, 2006).

Gram Staining

The procedure for Gram staining involved the application of the bacteria-laden smear on a glass slide followed by heat fixation before using crystal violet for one minute

of staining duration. The indole solution application lasted one minute, and then an acetone-ethanol mixture was used to decolorize the slide before counterstaining with safranin for one additional minute. The slide passed through air drying before being examined through light microscope testing (Tripathi & Sapra, 2020).

Motility Test

The motility test required scientists to inoculate bacterial strain into a motility agar before evaluating bacterial movement toward the point of application (Morales-Soto et al., 2015).

Catalase Test

Bacterial colonies received a 3% hydrogen peroxide (H_2O_2) solution that was placed onto a clean slide as part of the catalase test procedure. The bacterial strain showed catalase activity, as indicated by the formation of oxygen bubbles during the test (Reiner, 2010).

Oxidase Test

The experiment utilized 4.5 ml of nutrient broth to culture fresh bacterial samples. The assay contained 0.2 ml of 1% α-naphthol solution, followed by the addition of 0.3 ml of 1% p-aminodimethylaniline oxalate reagents. The test displayed positive for oxidase activity when it exhibited a visible color reaction through vigorous shaking movements (Shields & Cathcart, 2010).

Starch Hydrolysis Test

The starch test required plates to contain several streaked bacterial colonies positioned at the center prior to 37°C incubation for 48 hours. A starch hydrolysis detection test was performed with iodine solution addition after incubation (Sigmon, 2008).

Urease Test

A bacterial colony received placement on a urea agar plate before undergoing 48 hours to seven days of incubation at 35°-37°C. A positive urea test result indicated bacterial urease production through the appearance of pink color (Islam, 2016).

Antibiotic Susceptibility Testing

The Kirby-Bauer method was used for antibiotic testing to determine bacterial drug sensitivities during the procedure. Mueller-Hinton broth served to create a bacterial suspension which corresponded to the 0.5 McFarland standard. A standard distribution of suspension took place across Mueller-Hinton agar plates which received placement of antibiotic disks (Oxoid, UK) on their surface. The experiment used 37°C to incubate for 24 hours. The laboratory team evaluated bacterial antibiotic sensitivities by measuring antibiotic zone diameters in millimeters using standard methodology (Alhamadani & Oudah, 2022).

DNA Extraction and PCR

All bacterial isolates were enriched and the DNA was extracted using the Bacterial Genomic DNA Isolation Kit, a commercial Bacterial Genomic DNA isolation kit, ab288102, Thermo Scientific UK as per the manufacturer's instructions. The Thermo scientific spectrophotometer from the United Kingdom by using absorbance and percentage measurements helped to determine DNA concentration and its quality in extracted samples.

The identification of Bacillus subtilis was confirmed through the Polymerase Chain Reaction (PCR) since this technique amplifies the genes RecA and bsub. The Biotechnology Center produced the amplification primers as described in Table 1 (Pediaditakis, 2011). The PCR reaction comprised 25 µL solution containing 50 ng of the DNA template, 0.6 µM each of the primer and 12.5 µL of the master mix [1mmol/L MgCL2, 10mmol/L Tris, pH8, 500µg/ml BSA, 200 µmol/L each dNTP, 4 µmol/L

fluorescein, 1.25 units AmpliTaq Gold and 1.25 units Gold Taq DNA polymerase (Thermo Scientific, UK) and nuclease free water. These amplification reactions began with a denaturing step at 94°C for 4 minutes and then went through 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extending at 72°C for 45 seconds; the last step in the reaction was also a final extension at 72°C that lasted 4 minutes.

Bacteria	Primer Name	Sequence
Bacillus RecA gene	recA-F	5′-TGAGTGATCGTCAGGCAGCCTTAG-3′
	recA-R	5′-CYTBRGATAAGARTACCAWGMACCGC-3′
Bacillus bsub gene	Bsub-F	5′-CAAGAATGTCTAAGATCTCG-3′
	Bsub-R	5′-CAGTCGGGAAATCAGGC-3′

Sequencing of Bacillus Species

The laboratory technicians used Sanger sequencing in the Biotechnology Center to sequence the RecA gene PCR products after performing purification of the product. The nucleotide sequences of the PCR products were further analyzed using ChromasPro software, and the species identification was supported by BLAST analysis against GenBank sequences (Crossley et al., 2020).

Results

Morphological Identification of Bacillus Species

The bacteriological evaluation of intestinal wall samples from local broiler chickens involved an initial assessment of microbial morphology. The isolated bacteria exhibited characteristics of Gram-positive rod-shaped motile spore-forming microorganisms that match Bacillus species descriptions. The agar plate colonies displayed circular shapes and a lack of pigmentation, thereby confirming that the species existed as non-pigmented. Biochemical tests demonstrated that all isolates performed starch hydrolysis, which combined with positive results for catalyst and oxidase activities, yet showed no urease activity. The detection of specific morphological and biochemical traits revealed that the isolated strains belong to the Bacillus genus. The observed characteristics confirm previously documented descriptions of Bacillus species from (Cheesbrough, 2006).

Antibiotic Susceptibility Testing

Antibiotic susceptibility tests for *Bacillus subtilis* strains required disk diffusion analysis. The antibiotic susceptibility test showed that all isolated *Bacillus subtilis* strains reacted positively to multiple antibiotics, including trimethoprim-sulfa, kanamycin, ciprofloxacin, gentamycin, penicillin, and erythromycin. Laboratory measurements revealed that the inhibition zones of antibiotics measured at 25 mm for trimethoprim-sulfa and 20 mm for kanamycin, along with 30 mm for ciprofloxacin and 20 mm for gentamycin and penicillin (27 mm) and erythromycin (27 mm). The antibiotic susceptibility test results demonstrate *Bacillus subtilis* shows sensitivity to multiple standard antibiotics.

The bacterial isolates showed resistant traits against oxytetracycline colistin sulfate and cefotaxime with clavulanic acid and ampicillin. The observed antibiotic resistance stands as a potential problem in light of expanding antibiotic use in veterinary practices. Multiple studies involving Bacillus subtilis have documented comparable resistance patterns with results from this investigation (Mingmongkolchai & Panbangred, 2018).

Molecular Identification of Bacillus Species

The isolated bacterial strains underwent additional verification through conventional PCR that targeted the identification of the RecA gene and bsub gene sequences. **Figure 1**, together with **Figure 2**, shows the results of PCR amplification products. The reaction detected an 880 base pair (bp) fragment of the RecA gene that matched the expected dimensions for Bacillus species (Figure 1). The bsub gene amplification generated a 290 base pair fragment that matched the expected size described by Wattiau et al. (2001) (Figure 2).

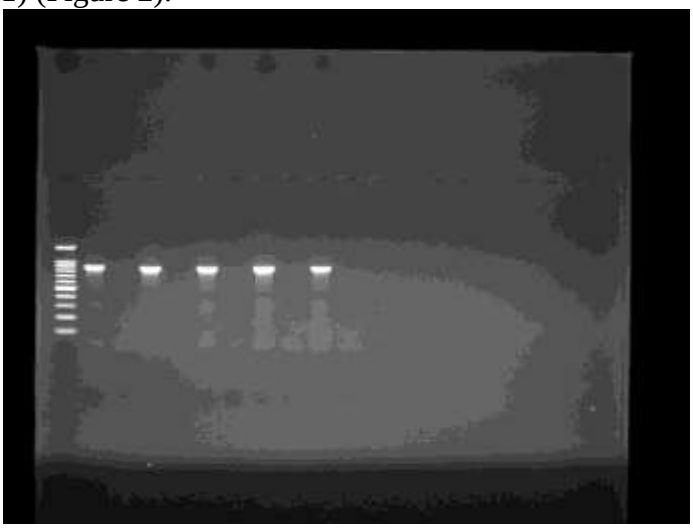

Figure 1: Amplification of the RecA gene of Bacillus spp. The target gene is the 16S rRNA gene, and the amplified fragment size is 880 bp.

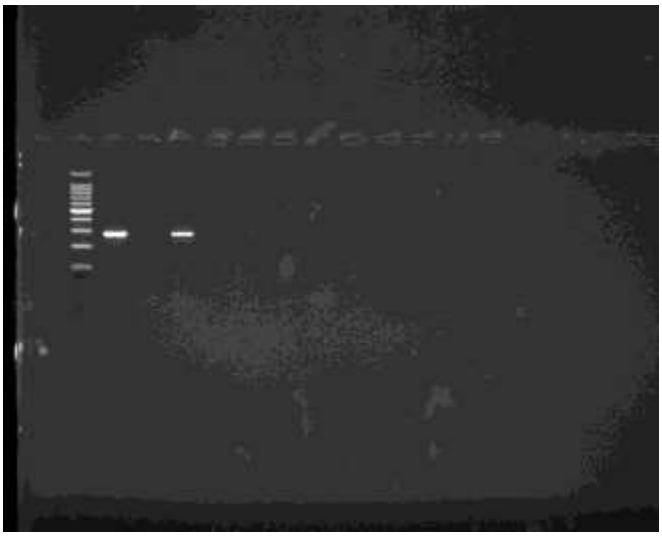

Figure 2: Amplification of the bsub gene. The size of the amplified fragment is 290 bp, as indicated by Wattiau et al. (2001).

The obtained RecA gene sequence underwent sequencing analysis, which included a database comparison from GenBank resources. Sequence analysis confirmed that Bacillus subtilis strain BMS had 97% identity with Bacillus subtilis strain AF and 98% identity with *Bacillus subtilis* strain AR, thus verifying the bacterial species' identification. A representation of the RecA gene sequence appears in **Figure 3**. The Bacillus subtilis strain FDAARGOS 606 complete genome sequence matched the isolates at a level of 97%, indicating successful molecular identification of the bacteria.

A-F

TGTCCCTAACATAGAAACAGTTCGGCAAGGTTCTATTATGAACTGGGAGAAA
GACAGATACAAGAATTTCTACTGTCCCAAGCGGCTCCCTCGCTCTTGATACAG
CACTAGGAATTGGCGGATATCCGCGCGGACGGATTATTGAAGTATATGGTCC
TGAAAGCTCAGGTAAAACAACTGTGGCGCTTCATGCGATTGCTGAGGTTCAG
CAGCAGGGCGGACAAGCCGCGTTTATCGATGCGGAGCATGCGTTAGATCCGG
TATACGCGCAAAAGCTTGGAGTCAATATCGAAGAGCTTTTACTGTCTCAGCCT
GACACAGGTGAGCAGGCGCTTGAAATTGCGGAAGCGCTGGTTCGAAGCGGTG
CAGTTGACATTGTTGTTGTCGACTCTGTGTTTTGTTTTTGTTCCGAAAGCGGAA
ATTGAAGGCGACATGGGAGATTCGCATGTTTTTTTTACTATGCTCGCTTAATG
TCTCAAGCGCTTCGTAAGCTTTCAGGGGCCATTAACAAATCGAAGACAATCG
CGATTTTCATTAACCAAAATTCGTGAAAAAAGTCGGCGTTATGTTCGGGAAC
ACCGGAAACAACTCCCTGGCGGGCCGCG

A-R

TTGCGAAATTCAAGTTCAGTTCAAGATCATGATTTCGCCTTCTTTTGAGATGC
CTTCTCCGTACATAATGTCAACCTCGGCTGTACGGAACGGCGGAGCTACCTTG
TTTTTCACGACTTTGATTTTCGTTTTGTTCCCCATTACGTCGTTGCCTTGTTTCA
GCTGTTCAGCACGGCGCACTTCAAGACGCACGGAAGAGTAGAATTTCAGCGC
GCGGCCGCCAGGAGTTGTTTCCGGGTTCCCGAACATAACGCCGACTTTTTCAC
GAATTTGGTTAATGAAAATCGCGATTGTCTTCGATTTGTTAATGGCCCCTGAA
AGCTTACGAAGCGCTTGAGACATTAAGCGAGCTTGTAAACCGACATGCGAAT
CTCCCATGTCGCCTTCAATTTCCGCTTTCGGAACGAGAGCCGCCACAGAGTCG
ACAACAACAATGTCAACTGCACCGCTTCGAACCAGCGCTTCCGCAATTTCAA
GCGCCTGCTCACCTGTGTCAGGCTGAGACAGTAAAAGCTCTTCGATATTGACT
CCAAGCTTTTGCGCGTATACCGGATCTAACGCATGCTCCGCATCGATAAACG
CGGCTTGTCCGCCCTGCTGCTGAACCTCAGCAATCGCATGAAGCGCCACAGT
TGTTTTACCTGAGCTTTCAAGACCATATACTTCAATAATCCGTCCGCGCGGAT
ATCCGCCAATTTCCTAGTGCTGTATCAGAGCGAGGGAGCCGCTTGGGACAGT
AGAAATTTCTTGTATCTGTCTTTTCTCCCAGTTTCATAAT

Figure 3: Sequence of the RecA gene. The obtained sequence shows 97% identity with Bacillus subtilis strain AF and 98% identity with Bacillus subtilis strain AR, confirming the isolates as Bacillus subtilis.

A BLAST analysis compared the sequence of the RecA gene against nucleotide database records in GenBank. The sequence comparison showed that the tested isolates matched 97% with *Bacillus subtilis* strain AF and 98% with *Bacillus subtilis* strain AR. The DNA sequence analysis of *Bacillus subtilis* strain FDAARGOS 606 validated the previous identification results, confirming the isolates belonged to *Bacillus subtilis*.

Discussion

This research examined Bacillus subtilis strains found in local broiler chickens that inhabit the intestinal region while testing their antibiotic resistance to determine their suitability as probiotics in poultry farming systems. The investigation revealed significant information regarding the biochemical properties of *Bacillus subtilis* strains collected from poultry alongside their resistance patterns. The successful outcomes demonstrated Bacillus subtilis shows valid probiotic potential, yet its antibiotic resistance capabilities remain a crucial safety concern for its employment as a probiotic in animal farming.

Morphological and Biochemical Characterization

Microbiological analysis and morphological and biochemical tests validated the Bacillus subtilis identity of bacterial strains extracted from broiler chickens' intestinal tracts. The Bacillus subtilis isolates demonstrated essential Bacillus genus traits through their Gram-positive rod shape with spore-forming capability (Cheesbrough, 2006). The bacterial

isolates displayed fundamental *Bacillus subtilis* features by showing motility patterns alongside oxidase activity and producing catalase enzyme. Test results showing a lack of urease activity match what (Sharma & Mallubhotla, 2022) previously identified as *Bacillus subtilis*. The bacteria identification was validated through a starch hydrolysis test because Bacillus subtilis demonstrates starch hydrolysis, differentiating it from other Bacillus species.

These results match the established biochemical patterns of *Bacillus subtilis*, according to (Mingmongkolchai & Panbangred, 2018). The microbiological properties matched Bacillus subtilis identification criteria, which makes these isolates viable candidates for usage as poultry farming probiotics.

Antibiotic Susceptibility Profile

The antibiotic susceptibility tests demonstrated that Bacillus subtilis showed sensitivity towards six clinically significant antibiotics: trimethoprim-sulfa, kanamycin, ciprofloxacin, gentamycin, penicillin and erythromycin. The antibiotic susceptibility tests demonstrated *Bacillus subtilis'* sensitivity to all tested agents, recommending their application in areas free from antibiotic requirements for therapeutic interventions. These strains show exceptional value for antibiotic use in animal or human treatment due to their susceptibility to penicillin and erythromycin, which indicates these bacteria would not compromise the effectiveness of these antimicrobial drugs (Adimpong et al., 2012).

The antimicrobial susceptibility testing revealed high level resistance towards oxytetracycline, colistin sulphate, cefotaxime with clavulanic acid and amoxicillin. This usage of these antibiotics might be restricted for animals because resistance development reduces the number of treatment options for animal infections. The studies also highlight the concerns involving the resistance towards oxytetracycline and colistin sulfate as both these medicines are commonly used in poultry farming for both, preventive and treatment purposes. Poultry harboring antibiotic-resistant strains in their gut come with complicated disease control challenges that could expose, and possibly reinforce, antibiotic-resistant bacteria into the food chain.

The observed antibiotic resistance patterns match prior studies, which show that Bacillus subtilis develops resistance to tetracyclines and ampicillin (Mingmongkolchai & Panbangred, 2018). A well-established scientific fact shows antibiotic resistance gene exchange between probiotics and pathogenic bacteria, but additional research should study horizontal gene transfer, specifically within Bacillus subtilis (Stenfors Arnesen et al., 2008).

Molecular Identification

Molecular confirmation of Bacillus subtilis occurred through PCR amplification of the RecA and bsub genes. Figures 1 and 2 show PCR amplification products with sizes that match expected target gene ranges, verifying the Bacillus subtilis nature of isolated microbes. The molecular identification through RecA gene sequence analysis identified the microbe as Bacillus subtilis strain AF with 97% similarity and Bacillus subtilis strain AR with 98% similarity. The results support the molecular identification methodology demonstrated by the genomic sequence of Bacillus subtilis strain FDAARGOS 606 (Bakri, 2023).

The identical RecA gene sequence between this study and known Bacillus subtilis strains validates PCR as a rapid Bacillus identification tool, which is valuable for probiotic research. Previous studies prove RecA gene effectiveness as a bacterial species identification marker that offers enhanced precision compared to 16S rRNA sequencing methods (Bakri, 2023).

Implications for Probiotic Use

This research provides valuable insights regarding using Bacillus subtilis probiotics for poultry farming applications. The antibiotic-resistant strains isolated during this study expose potential risks to the sustainable and safe usage of Bacillus subtilis probiotics in animal husbandry despite its documented growth-promoting and gastrointestinal protective properties in poultry. The transfer of antibiotic-resistance genes from probiotic strains into pathogenic gut bacteria leads to complications in poultry infection management. It increases the transmission of resistant bacteria from poultry to human consumers through the food supply chain (de Mesquita Souza Saraiva et al., 2022).

Monitoring probiotic antibiotic resistance profiles needs consistent maintenance to stop the strains from spreading antibiotic-resistant bacteria to animal and human populations. *Bacillus subtilis* antibiotic resistance mechanisms require additional study and research on how animal production systems affect gene transfer between probiotics and pathogens. The research outcomes underpin the need for complete knowledge about probiotic and antibiotic interactions across animal agriculture because it protects animal and human well-being.

Conclusion

This investigation revealed significant findings regarding broiler chicken's *Bacillus subtilis* isolates' morphological and biochemical features, molecular properties, and susceptibility to antibiotics. The promising probiotic properties of these strains require poultry farmers to watch for potential antibiotic resistance issues when *Bacillus subtilis* is used as a probiotic. Additional research must explore antibiotic resistance mechanisms and appraise potential safety concerns related to antibiotic-resistant probiotic utilization in animal farming systems.

Recommendations

The efficient and safe implementation of Bacillus subtilis as an animal agriculture probiotic demands studies measuring strains' antibiotic sensitivities in all animal production environments. Assessing Bacillus subtilis as a probiotic in animal agriculture requires investigating potential safety hazards linked to antibiotic resistance. Strict medical protocols should be adopted for antibiotic-resistant probiotic strain use throughout treating bacterial animal infections when probiotic feed exists in the environment. Additional scientific studies should investigate antibiotics' effects on probiotic bacteria in commercial animal farming operations. The evaluation and monitoring of antibiotic susceptibility profiles among commercially used probiotics must be performed routinely to maintain safety standards and effectiveness while preventing animal health risks and stopping antibiotic-resistant bacteria from spreading in agricultural operations.

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