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RESEARCH ARTICLE

Whole genome sequencing of *Bacillus paranthracis* isolated from commercial white bread dough

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Abstract - Bread is a widely consumed staple food, and ensuring its microbiological safety is essential due to its high consumption across diverse population groups. Although commercial bread production relies primarily on yeast fermentation, other microorganisms may be introduced unintentionally through raw materials, handling practices, or processing environments. In this study, *Bacillus* species associated with bread dough were isolated and characterized to evaluate their potential food safety relevance using whole-genome sequencing. The bacterial isolates were cultured, and genomic DNA was extracted, followed by the library and sequencing using the Illumina NovaSeq 6000 platform. Whole-genome analysis identified the isolate as *Bacillus paranthracis*, a member of the *Bacillus cereus* group previously associated with foodborne illness. The draft genome assembly had a total size of 10.3 Mb with a GC content of 49.1%. Functional annotation revealed genes associated with metabolism, stress response, and cellular processes, as well as genomic features linked to virulence and antimicrobial resistance. The detection of *B. paranthracis* in bread dough highlights the importance of monitoring microbial communities within dough matrices, as certain members of the *B. cereus* group may pose potential food safety concerns. These findings demonstrate the value of whole-genome sequencing as a powerful tool for assessing the safety relevance of bacterial contaminants in food systems and provide insights that may support improved hygiene practices and risk assessment in bread production.

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1. Introduction

Bread is a staple food consumed by most people worldwide, and its history dates to the early civilisations when cereal grains were domesticated [1]. White bread is one of its varieties and is very popular for its soft texture, mild flavour, low cost, and convenience. The production of bread in modern food systems is an industrial process and is categorised as a ready-to-eat food, with its microbiological safety a vital element of food safety and human health [2]. Even though bread production is mainly dependent on the controlled fermentation process of *Saccharomyces cerevisiae*, the processing environment of bread dough is not sterile. Microorganisms can be accidentally introduced by raw materials, handling procedures, equipment, or processing conditions. Although fermentation and baking reduce the number of microbes surviving in bread matrices, some bacteria can persist. Specifically, representatives of the genus *Bacillus* are frequently associated with cereal-based products and bakery settings because of their ubiquity and ability to form heat- and stress-resistant endospores [3]. These spores allow bacteria to survive in unfavourable conditions that can arise during dough preparation and d baking, and some species may remain in the final product.

Foodborne illness is an issue of concern across the world, and in Malaysia, there are food poisoning cases recorded every year [4]. Spores-forming bacteria such as *Bacillus* spp. in staple foods represent a potential food safety risk, as some members of the *Bacillus cereus* group can produce toxins and other harmful metabolites [5]. Nevertheless, standard microbiological techniques may not be able to distinguish closely related *Bacillus* sp. with sufficient accuracy, thereby limiting the feasibility of proper risk evaluation. With the development of whole-genome sequencing (WGS), especially on Illumina platforms, microbial identification and characterisation have been significantly enhanced. WGS enables accurate taxonomic classification and detailed analysis of genetic characteristics across metabolism, stress resistance, virulence, and antimicrobial resistance [6]. Despite its value, there is a relative lack of genomic data on bacterial contaminants in white bread dough in Malaysia.

Therefore, this study aims to isolate and identify *Bacillus* sp. from raw dough, perform molecular characterisation through DNA extraction, library preparation, and whole-genome sequencing, and elucidate the functional properties of the isolate in the context of food safety, with the hope of providing genomic insights that support better monitoring, risk evaluation, and hygiene management in bread production systems.

2. Materials and Methods

2.1 Sample Collection, Preparation and Bacterial Cultivation

Raw dough of commercial classic white bread was obtained directly from the manufacturer's facility in Shah Alam, Selangor, and stored at -20°C until analysis to halt fermentation and prevent microbial overgrowth. Bacterial isolation was done under aseptic conditions through serial dilution and culture-based methods. A slurry of a homogenised portion

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of dough in sterile distilled water was prepared, and subsequently diluted fivefold (10^{-1} to 10^{-5}). Aliquots (100 µL) of each dilution were placed on De Man, Rogosa, and Sharpe (MRS) agar plates prepared according to the manufacturer's instructions and incubated anaerobically at 37°C for 48 h. After incubation, plates with countable colonies were chosen. Isolations were further performed by subculturing distinct colonies using the quadrant streaking technique on fresh MRS agar until single colonies of morphologically homogeneous bacteria were isolated. The selected isolates were inoculated into nutrient broth to enrich them before downstream analyses. Gram staining was done to identify the cell wall properties of the purified isolate. In short, a smear was placed on the glass slide, heat-fixed, and stained in series with crystal violet, iodine, and ethanol before safranin. The stained cells were observed under a light microscope to identify the isolate using the Gram reaction and the cell morphology.

2.2 DNA Extraction and Molecular Identification

The single bacterial isolate was cultured overnight in MRS broth. Genomic DNA (gDNA) was isolated from 3 mL of culture using the NucleoSpin Microbial DNA Kit following the protocol for Gram-positive bacteria. Briefly, mechanical lysis was performed in a swing mill for 12 min using MN Bead Tubes Type B. A 100 µL of Buffer BE was used to elute the DNA. The Nanodrop spectrophotometer was used to determine concentration and purity, while DNA integrity was assessed by 1% agarose gel electrophoresis [7].

Universal primers targeting the V3 region were used to amplify the bacterial 16S rRNA gene, enabling preliminary taxonomic identification [8]. The polymerase chain reaction (PCR) was performed on an Eppendorf Mastercycler using a standard thermal cycling programme. PCR amplification, followed by DNA extraction, was used to amplify the template and assess extraction success. The bacterial 16S rRNA gene was amplified by PCR using an Eppendorf thermal cycler with the following conditions, with modifications [9]. The PCR products were subjected to electrophoresis on a 2% agarose gel, and a 100 bp DNA ladder was included to accurately estimate the size of the amplified fragments. PCR products were then subjected to Sanger sequencing, which was outsourced to Apical Scientific Sdn. Bhd. The sequence data were applied to identify the bacterial isolate at the genus level.

2.3 Library Preparation and Whole-genome Sequencing

Mechanical homogenisation of the bacterial culture with 0.1 mm glass beads and centrifugation at 10,000 × g for 5 min were performed according to the manufacturer's protocol for the ZymoBIOMICS DNA Extraction Kit to extract genomic DNA. The amount and quality of the DNA were determined through the Qubit dsDNA High Sensitivity assay (Thermo Fisher Scientific). About 100 ng of genomic DNA was bisected to 350 bp or so using a Bioruptor and placed to be sequenced using NEBNext Ultra II DNA Library Preparation Kit (New England Biolabs). Paired-end sequencing (2 ×150 bp) was performed on an Illumina NovaSeq 6000 platform, producing approximately 1 GB of raw sequencing data.

2.4 Deep Genome Data Analysis

Quality checking of raw reads was done by SeqKit v2.3.0 [10] and the pre-processing of adapter trimming and error correction by Trimmomatic v0.39 [11] and Lighter v1.1.3 [12], respectively. The de novo assembly was performed using high-quality reads with SPAdes v4.0 [13], and plasmid-associated contigs were identified with GeNomad v1.8.1 [14]. Assembly numbers were analysed using SeqKit v2.3.0 statistics, and genome completeness was assessed using BUSCO v5.4.3 with the *Bacillus paranthracis* dataset [15]. Rapid Annotation using Subsystem Technology (RAST) was used for functional genome annotation, enabling classification of genes by metabolism, stress response, sporulation, and cellular processes. Automated Multi-Locus Species Tree (AutoMLST2) was used to generate a multilocus sequence-based species tree via phylogenetic placement. The genome was analysed using tools from the Centre for Genomic Epidemiology to assess the presence of antimicrobial resistance genes and virulence factors, as well as other genome determinants relevant to food safety.

3. Results and Discussion

3.1 Isolation of a Single Bacterial Colony

A serial dilution of the raw dough suspension was prepared, and aliquots were spread onto nutrient agar plates to obtain isolated colonies. Following incubation, two distinct colony morphologies were observed on the 10^{-1}dilution plate. Colony type A exhibited a filamentous appearance with flat elevation, irregular margins, and a whitish, rough, dry, and wrinkled surface texture. The filamentous morphology suggested that this colony type was likely fungal in origin, which is consistent with the presence of yeasts and filamentous fungi commonly associated with dough fermentation environments [16]. In contrast, colony type B displayed a smooth morphology with entire margins and raised elevation. The colonies appeared whitish, smooth, and moist with a relatively large size, characteristics more typical of bacterial growth [17]. Given the objective of isolating bacterial species associated with the dough matrix, colony type B was selected for further purification and characterisation. A representative colony of type B was picked and streaked onto fresh agar plates using the quadrant streaking method to obtain isolated bacterial colonies. After incubation (Day 1), the streaked plate produced colonies that were irregular to slightly filamentous in shape, with entire to slightly undulate margins and flat to slightly raised elevation. The colonies exhibited a creamy to off-white colouration and a smooth to slightly rough surface texture, with relatively large colony size.

To ensure purity of the isolate, a single representative colony from the streaking plate was further sub-cultured onto a new agar plate (Day 2). The resulting colonies displayed a circular to irregular morphology with entire to slightly undulate margins and flat to slightly raised elevation. The colonies maintained a creamy to off-white colouration, a smooth to

slightly rough texture, and a medium to large colony size. The consistency of colony morphology across successive culturing steps indicated that a stable and purified bacterial isolate had been obtained [18], which was subsequently subjected to genomic DNA extraction and whole-genome sequencing for downstream molecular characterisation (Table 1).

Table 1. Observation on streaked agar plates for 2 days

Agar plates	Characteristics				
	Shape	Margin	Elevation	Colour & Texture	Size
B A 10^{-1} agar plate	A: Filamentous B: Smooth	A: Filamentous B: Entire	A: Flat B: Raised	A: Whitish, rough, dry, and wrinkled B: Whitish, smooth, and moist.	A: Large B: Large
Day 1(B) Streaking plate	Irregular and filamentous	Entire to slightly undulate	Flat to slightly raised	Creamy to off-white, Smooth to slightly rough	Large
Day 2(B) Sub-cultured plate	Circular to irregular	Entire to slightly undulate	Flat to slightly raised	Creamy to off-white, Smooth to slightly rough	Medium to large

Table 2. Gram staining under the microscope

Gram staining under the microscope	Characteristics	
	Type of Bacteria	Shape
Streaking plate (B) Day 2 (100x magnification)	Gram-positive	Rod-shaped
Enrichment Broth (B) (100x magnification	Gram-positive	Rod-shaped

3.2 Gram Staining

The Gram staining analysis was conducted to determine the cellular morphology and Gram reaction of the bacterial isolate obtained from the dough sample (Table 2). Microscopic examination of the stained smear prepared from the Day 2

streaking plate (B) under oil immersion (100× objective) revealed that the bacterial cells retained the crystal violet stain, appearing purple under the microscope, which is characteristic of Gram-positive bacteria. The cells exhibited a rod-shaped (bacillary) morphology, commonly arranged singly or in short chains. This staining pattern indicates the presence of a thick peptidoglycan layer in the bacterial cell wall, which retains the primary stain during the decolourisation step of the Gram staining procedure [19]. Similarly, microscopic observation of the smear prepared from the enrichment broth culture (B) showed consistent results, with cells displaying Gram-positive rod-shaped morphology. The uniform Gram reaction and morphology observed in both the solid culture (streaking plate) and liquid enrichment culture suggest that the isolate maintained stable phenotypic characteristics during cultivation, supporting the likelihood that the culture represented a single dominant bacterial population rather than a mixed community.

The observed morphology is consistent with members of the *Bacillus* genus, which are typically Gram-positive, rod-shaped, and capable of forming endospores. Species belonging to the *Bacillus cereus* group commonly display similar microscopic characteristics, including large rod-shaped cells that may appear singly or in chains. In food matrices such as dough, Bacillus species can originate from raw materials, flour contamination, or processing environments, owing to their ability to survive harsh conditions via spore formation [20]. Although Gram staining provides important preliminary evidence regarding bacterial classification, it is insufficient for precise species-level identification because several *Bacillus* species share similar morphological and staining characteristics. Therefore, the Gram-positive, rod-shaped morphology observed in this study provided an initial indication that the isolate likely belonged to the *Bacillus* lineage.

3.3 DNA extraction and Sanger Sequencing

Genomic DNA was extracted from the purified bacterial isolate, and its integrity was verified using 1% agarose gel electrophoresis, which showed a clear DNA band. DNA concentration and purity were first assessed using a NanoDrop spectrophotometer, which showed two samples with suitable concentrations (87.2 ng/μL and 103.2 ng/μL) and A260/A280 ratios of 1.97 and 1.84, indicating good DNA quality. One sample with very low concentration (3.7 ng/μL) was excluded from further analysis. DNA quantification was further confirmed using a Qubit 4 Fluorometer, which produced comparable concentrations (97.6 ng/μL and 97.8 ng/μL). The 16S rRNA gene was amplified by PCR and visualised on a 2% agarose gel, producing a distinct band of approximately 300 bp. To obtain a more accurate taxonomic identification and to evaluate the potential food-safety relevance of the isolate, Sanger sequencing was subsequently performed. The genomic analysis later confirmed that the isolate corresponded to *Bacillus paranthracis*, a species within the *Bacillus cereus* group that has been previously associated with food contamination and foodborne illness [21].

3.4 Whole-Genome Sequence and Phylogenetic Analysis

The assembled genome of isolate QS-AS1 was 10.3 Mb, which is larger than the reported genome size of members of the *Bacillus cereus* group (5.2 -6.5 Mb). This implies that the assembly is a draft genome and may contain redundant regions, assembly redundancy, or plasmid-derived contigs [20]. The GC content measured 49.1, which aligned with values for *Bacillus paranthracis* and related species, confirming proper taxonomic classification [22]. Assembly metrics indicated good contiguity and broad genome coverage, with an N_{50} of 918,959 bp, 67 contigs, and 10,956 predicted coding sequences.

AutoMLST2 reveals the Mash distance, Average Nucleotide Identity (ANI), P-value, Genus, Order, and type strain of the query organisms that are genetically similar to them. Mash distances are established through the reduction of large sequences to small, representative sketches [23]. ANI is one of the highest-quality standards for species circumscription in digital whole-genome comparisons [24]. Using AutoMLST2, whole-genome analysis showed that QS-AS1 was most closely related to *Bacillus paranthracis*, with a low Mash distance of 0.0357, indicating that these species are highly genetically similar. Other related species, such as *B. pacificus*, *B. anthracis*, and *B. tropicus*, were found to have greater Mash distances. The presence of *Ralstonia pickettii* in the initial Mash hits was attributed to k-mer-based screening artefacts, which were resolved using Average Nucleotide Identity (ANI) and a phylogenetic tree [23]. ANI analysis revealed a 96.4% similarity between QS-AS1 and *B. paranthracis*, which is above the accepted species threshold of 95-96% [25], whereas ANI values for other closely related *Bacillus* species ranged from 92.5% to 94.1%. Considering the high genomic similarity often observed among members of the *B. cereus* group, ANI analysis would be highly significant for resolving closely related taxa within this complex (Figure 1). This classification was further supported by a multilocus sequence phylogeny showing that QS-AS1 was tightly clustered with the B. paranthracis type strain, with high support (0.999975). The isolate was found to form a clear clade with other members of the B. cereus sensu lato group, including B. cereus, B. thuringiensis, and B. anthracis, and with a suitable outgroup (Rhizobacter sp.) located at the root of the tree. Altogether, Mash distance, ANI, and phylogenetic studies make the case of QS-AS1 as Bacillus paranthracis, a member of the B. cereus sensu lato group.

3.5 Factors Attributed to Food Safety

A total of 437 subsystems associated with metabolism, stress response, and defence were identified by functional annotation, providing sufficient genomic resolution for downstream food safety evaluation (Figure 2). RAST-based functional annotation revealed that most genes were associated with core metabolic functions, stress response, and sporulation, which collectively contribute to the environmental resilience of *Bacillus* species and their ability to survive under food-processing conditions.

Although most of the annotated subsystems were involved in cell maintenance rather than virulence, further screening identified genes involved in resistance, with several responsible for resistance to antibiotics and toxic compounds.

Aminoglycosides, tetracycline, β-lactam, streptothricin, and heavy metals (e.g., copper, cadmium, cobalt, and mercury) resistance genes were identified. A few of the resistance genes have a phenotype associated with the streptomycin resistance gene, which are *aph(6)-Id* and *aph(3")-lb*, giving them the ability to survive and grow in the presence of the antibiotic streptomycin [26]. Meanwhile, other resistance genes include *blaOXA-22*, which is associated with ampicillin resistance; *blaOXA-60b*, which is associated with an unknown beta-lactam [27]; *sul1, which* is associated with sulfamethoxazole [28]; and *tet(C)*, which is associated with tetracycline [29]. It is important to note that the sequence identity of the resistance genes *aph(6)-Id, aph(3) -Ib,* and *sul1* was 100%, implying that they are highly conserved genetically.

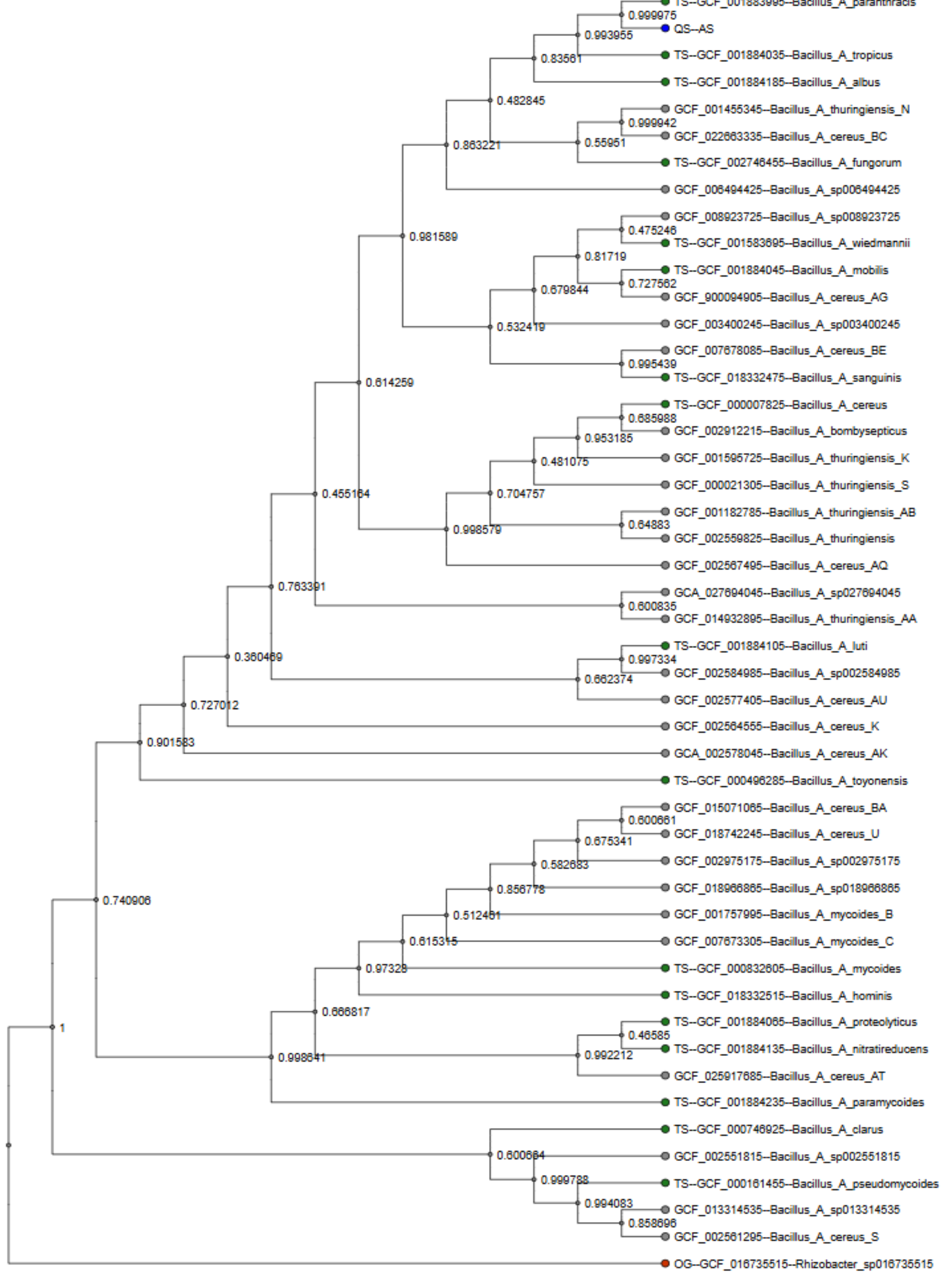

Figure 1. The phylogenetic tree generated by autoMLST QS-AS1 is the query sequence of the isolate

Genomic analysis also identified prophage-related factors involved in phage replication and integration, indicating past horizontal gene transfer events. No plasmid-associated functions were identified, indicating that the major source of genome plasticity in this isolate is phage [30]. Moreover, a LIPI-1-like pathogenicity island was identified, underscoring the possibility that virulence-related genes were acquired via horizontal gene transfer [31]. It is interesting to note that a LIPI-1-like region has been identified, and *Listeria monocytogenes* encodes virulence factors that help it invade host cells, survive within them, move along actin, and attach to other cells [32]. These characteristics indicate the need to monitor *Bacillus anthracis* in food environments, although it is not a pathogen; however, its genomic plasticity and food safety implications remain a concern.

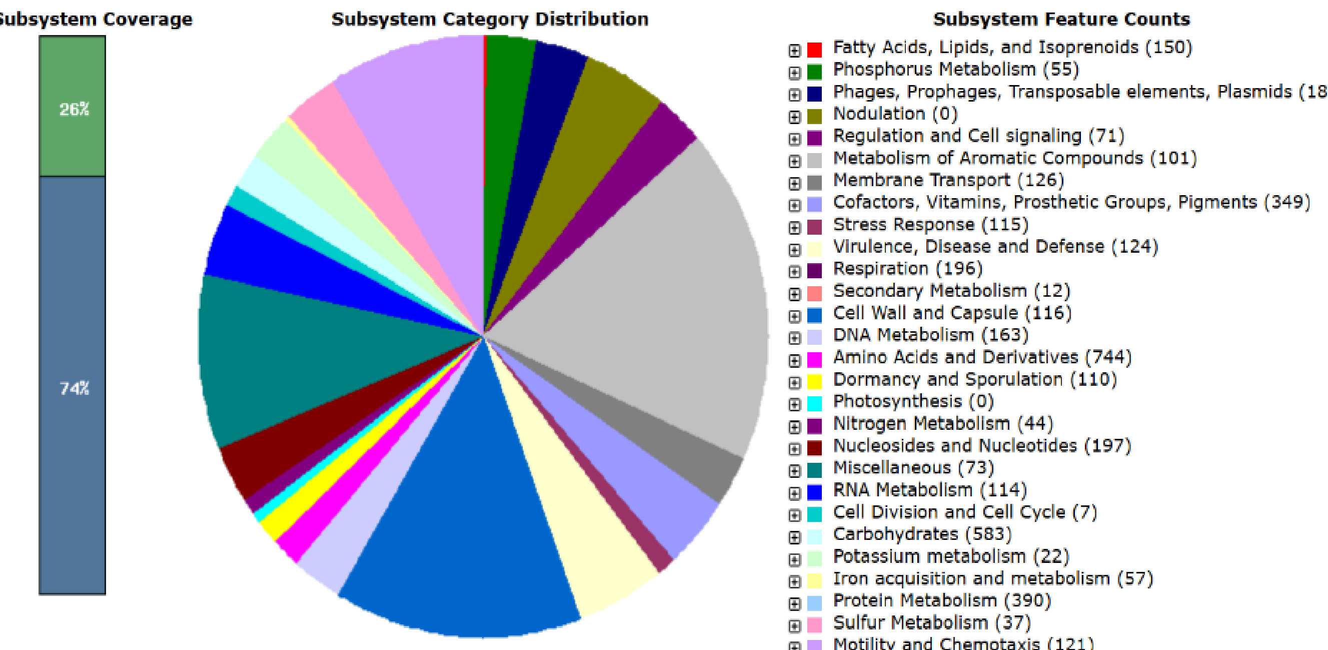

Figure 2. Gene annotation based on RASTk subsystems

4. Conclusions

This study successfully isolated and identified *Bacillus paranthracis* from commercial bread dough and characterised the isolate using whole-genome sequencing. Although the genome assembly was at a draft level, it provided insight into the organism's adaptability and potential relevance to food safety. Genomic analysis revealed genes associated with stress responses, sporulation, and survival in harsh environments, supporting its persistence in food-processing settings. The presence of virulence-associated and antimicrobial resistance genes, together with mobile genetic elements such as prophage regions and a *LIPI-1*–like genomic island, suggests genomic plasticity and potential adaptive advantages. These findings highlight the importance of monitoring spore-forming *Bacillus* species in bread and other cereal-based foods, while further phenotypic studies are needed to better evaluate their food safety implications.

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Conflict of Interest

I would like to declare no conflicts of interest.

CRediT Author Statement

Hajar Fauzan Ahmad: Conceptualisation; Methodology; Validation; Formal analysis; Resources; Supervision; Project administration; Writing - review & editing; Funding acquisition.
Zi Qing Tan: Methodology; Investigation; Formal analysis; Data curation; Software; Validation; Visualisation; Writing - original draft; Writing - review & editing.
Nur Aisyah Sumaiyah Mohamad Faizal: Methodology; Investigation; Formal analysis; Data curation; Software; Validation; Visualisation; Writing - original draft; Writing - review & editing.
Nazmi Harith-Fadzilah: Methodology; Investigation; Formal analysis; Data curation; Software; Validation; Visualisation; Writing - original draft; Writing - review & editing.

Ethics Statement

This study did not involve human participants or animal subjects. Ethical approval was therefore not required for this research.

Declaration of Generative AI in Scientific Writing

During the preparation of this work, the authors used QuillBot to improve sentence structure and grammar. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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