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Inhibitory Effects of *Bacillus subtilis* Isolated from Meju (Fermented Soybean Brick) on the Growth of *Aspergillus parasiticus*

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Abstract: Background: Meju is a base material for making soy sauce, soybean paste, and red chili pepper paste, which are representative ingredients of Korean cuisine. Objectives: This study aimed to isolate a predominant bacterial strain of *B. subtilis* from meju and to observe its inhibitory effects on an aflatoxigenic mold. Methods: We used yellow soybeans (*Glycine max (L.) Merr.*) grown in South Korea, and meju was produced according to the recommended methods of the Korea Food Research Institute. The identification of the strain was conducted based on its morphological and biochemical characteristics and 16S rDNA sequence. Evaluation of the bacterial effect against *A. parasiticus* ATCC 15517 was done in yeast extract–sucrose broth at 28 °C. Its inhibitory effect was evaluated using two approaches: mycelial weight and aflatoxin production. Aflatoxins were determined using high-performance liquid chromatography. Results: In the meju samples fermented for three months, a *B. subtilis* strain, K-0924 was identified. At the end of the incubation period of *A. parasiticus*, dry mycelial weight was significantly reduced by more than 80% ($p < 0.01$) and total aflatoxin production was inhibited by more than 63% ($p < 0.05$) in the presence of *B. subtilis*. Conclusions: These results indicate that *B. subtilis* K-0924 inhibits the growth and aflatoxin production of toxigenic *Aspergillus*, which can be contaminated with meju. We could expect more inhibition by other bacteria related to fermentation of meju, and further examination is necessary on species other than *B. subtilis*.

Keywords: *Bacillus* species; *B. subtilis*; meju (fermented soybean brick); *Aspergillus parasiticus*

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1. Introduction

Fermented foods have a long history in Korea as in many other countries. Koreans traditionally enjoy many kinds of fermented foods such as doenjang (soybean paste), ganjang (soy sauce), gochujang (red chili pepper paste), kimchi (fermented vegetables), and sikhye (fermented rice beverage). They are consumed in daily meals and are representative essentials used in Korean cuisine, except sikhye. The beneficial effects of these fermented food in vivo and in vitro [1–4] and their food and nutritional properties have been the subject of scientific investigations for the number of years [5–7]. However, there are fewer studies on meju, which is the base of all *jang*, namely, doenjang, ganjang, and gochujang. Meju is a soybean brick that is made of boiled soybean seed.

The production process of meju consists of two stages: making meju bricks and fermentation of the bricks. In the first stage, soybean seeds are prepared by washing, soaking in water for several hours, boiling, and crushing. The crushed paste is shaped as a brick. In the next stage, the shaped brick is kept in contact with rice straw. The fermentation period is about three months, but varies depending on the environment of the drying room. In this drying period, the brick is fermented naturally. From the initial wet brick to the dried and completed brick, we collectively refer to them all as "meju". Although some microorganisms in soybeans might be killed by cooking, meju bricks could be contaminated with microorganisms in the environment during preparation and fermentation.

Although the microorganisms that cohabit during the fermentation of traditional Korean meju are very diverse and complex, several studies have been conducted to understand and determine the microorganisms involved in meju fermentation [8–12]. Microorganisms that may be involved in meju fermentation include various bacteria, such as *Bacillus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, and *Staphylococcus*, and molds, such as *Aspergillus*, *Rhizopus*, *Penicillium*, *Cladosporium*, and *Mucor* [13]. Among others, *Aspergillus* and *Bacillus* are suggested as the major fungi and bacteria, respectively, responsible for fermenting meju [13–15]. Although extensive research has been conducted to understand the combined effect of the mold and the bacteria during meju fermentation, it is difficult for us to completely interpret it, because it inevitably varies depending on the environment in which fermentation takes place.

Bacillus spp. have long been known to be common contaminants of human food, animal feeds, and the environment. It has been suggested that *B. subtilis* is the dominant species of microorganisms in soy sauce, soybean paste, and meju [16–18].

Certain strains of *Aspergillus*, including *A. flavus*, *A. parasiticus*, *A. nomius*, and *Aspergillus tamarii*, are known contaminants during the fermentation of soy products [19–22]. One family of mycotoxins, the aflatoxins produced by *A. flavus*, *A.parasiticus*, and *A. nomius*, are proven carcinogens and immunotoxins and cause growth retardation in animals [23,24]. Aflatoxins are also hazardous to public health because they are a human carcinogen [25]. The International Agency for Research on Cancer indicated aflatoxin as carcinogenic to humans and animals [26]. Aflatoxin contamination in food crops, agriculIural products, and feed is a global threat that compromises the safety of food and feed and influences the agricultural economy [27]. Aflatoxins are heat-stable and rarely degraded during cooking and processing. Therefore, various physical, chemical, and biological treatments have been proposed to control the growth of the toxigenic mold and aflatoxin production [28,29]. However, novel method or strategies have not been found yet. Therefore, aflatoxins in food and feed are legally regulated in more than 80 countries.

Although previous studies have reported on the antifungal activity of *Bacillus* spp. against aflatoxin-producing fungi such as *A. flavus* and/or *A. parasiticus* [19,30], further studies are still warranted to understand these features in more detail. In this study, we aim to obtain better understanding of meju and fermented soybean foods by investigating the competitive features of *Bacillus* and aflatoxigenic *Aspergillus*.

This study was conducted: (i) to investigate the changes in the dominant bacterial species responsible for the fermentation of soybeans into meju, (ii) to isolate the strain from the meju we made, and (iii) to investigate antifungal effect of the dominant strain derived from meju. The effect was studied by a combination of *B. subtilis* isolated from meju and *A. parasiticus*, which is a known fungal strain that produces aflatoxins.

2. Materials and Methods

2.1. Chemicals and Media

All reagents used in this study were analytical grade purity or better. Nutrient agar and other media were purchased from Difco Lab. (Detroit, MI, USA). Aflatoxin standards for HPLC injection were purchased from Supelco (Bellefonte, PA, USA). The standard solutions were diluted prior to analyses.

2.2. Preparation and Fermentation of Meiu Cakes

A soybean cultivar (*Glycine max (L.) Merr.*), *Hwanggeum*, grown in North Gyeongsang Province in South Korea was selected for this study. These yellow beans are harvested in the fall in South Korea, dried, and usually used to make meju and tofu. The proximate composition of the soybeans we used followed the Korean Food Composition Database of the National Academy of Agricultural Sciences, i.e, moisture 11.2%, ash 4.9%, crude fat 14.7%, crude protein 36.2%, and carbohydrates 33.0% [31].

In South Korea, traditional meju is usually recommended to be made in winter. The beginning of winter is called *Ipdong* (especially when the sun has reached the celestial

longitude 225°) and usually begins around November 7. Therefore, we prepared meju in the middle of November. Soybean seeds were cooked into meju bricks, and the preparation and fermentation methods for meju bricks in this study followed the recommendations of the Korea Food Research Institute [32]. Soybeans were selected, washed, and soaked in water for 12 h. They were drained for 1 h and then heated with steam at 10 psi for 1 h. The steamed soybeans were cooled to 40 °C, then crushed in a mortar. The paste was molded into a cubical shape, the so called meju brick. The size of each brick was 10 × 12 × 15 cm and wet weight was about 1000 g. The meju bricks were exposed to sunlight during the day and kept indoors at night for more than 3 days until they were well dried. After this process, crevices had formed on the surfaces of the meju bricks. The dried meju bricks were then fermented for 3 months in a dark place at 20 ± 2 °C. Each brick was separated by a tie of rice straw to aid/promote fermentation. The bricks were sampled at initial stage and investigated/observed every month for the determination of the microbial population.

2.3. Enumeration and Isolation of Bacteria

The population of aerobic mesophiles and *Bacillus* species in meju samples was determined at 0, 1, 2, and 3 months of fermentation in this study. A 10 g piece for each meju brick was taken and suspended in 90 mL sterile diluent containing 0.1% peptone, and 0.85% NaCl, with pH adjusted to 7.0, and shaken vigorously for 2 min. From appropriate tenfold dilutions, enumeration of aerobic mesophiles was carried out on plate count agar (Difco Lab., Detroit, MI, USA) incubated at 37 °C for 24–48 h. The population of *Bacillus* species was enumerated on the plate of nutrient agar (Difco Lab.) incubated at 30 °C for 3 days. To confirm that colonies were *Bacillus* species, they were examined by colony and cell morphology [33]. Gram reaction, and catalase test. Gram-positive, catalase-positive rods bearing phase-bright spores were determined as *Bacillus* species. The bacterial colonies were isolated according to their morphological characteristics. Individual colonies were randomly selected and purified by single colony isolation after re-streaking on Luria–Bertani (LB) agar medium.

2.4. Identification and Characterization of Bacillus Isolates

In this study, morphological characteristics of the *Bacillus* isolates were examined according to methods suggested by Sneath [34]. An API 50 CHB test kit (bioMerieux, Marcy l'Etoile, France) was used to assess for biochemical characteristics of the strain. The identification results were confirmed by referring to the program apiweb (https://apiweb.biomerieux.com; 1 January 2020) [35]. We performed biochemical tests, namely, fermentation of glucose, arabinose, mannitol, and xylose, hydrolysis of casein, gelatin, and starch, nitrate reduction, production of indole and acetyl methyl carbinol, and utilization of citrate. Testing of their growth in anaerobic conditions was conducted. We studied the ability of the *Bacillus* isolates to grow at temperatures of 5 °C, 20 °C, 30 °C, 50 °C, and 60 °C. The morphological characteristics of the isolated strain were observed with a phase-contrast microscope (Olympus, Tokyo, Japan, high-class system BX51) using cells grown on LB media.

2.5. Genome Sequencing

To determine the 16S rDNA sequences, the strain's genomic DNA was extracted, and PCR amplification and sequencing of the 16S rRNA gene were performed to confirm. Amplification was done with the Program Temp Control System (ASTEC PC708, Japan). The genomic DNA of an isolated *Bacillus* strain was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), following the standard protocol for Gram-positive bacteria. Prior to genome sequencing, the quality of the isolated DNA and the molecular identity was confirmed by the sequencing of the 16S rRNA gene. A sequence ranging from nucleotide 9 to nucleotide 1523 of the 1556 bp 16S rRNA gene (97.4%) was amplified with the universal primers 785F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 907R (5′-CTACGGCTACCTTGTTACGA-3′). Amplified fragments were purified and

sequenced using the same primers. An identity score of 99% was considered identification at genus and species levels, respectively, according to NCBI BLAST (https://www.ncbi.nlm.nih.gov/ accessed on 31 December 2023) [36].

2.6. Inoculum Preparation

A fungal inoculum was prepared from single-spore cultures of *A. parasiticus* ATCC 15517. The strain was grown on potato–dextrose agar (PDA) (Difco Lab., Detroit, MI, USA) in Petri plates for 10 days at 28 °C. Spores were washed from the plates with sterile distilled water containing 0.1% Tween 80. The concentration of dislodged spores was determined with a hemacytometer and diluted to 10^6 conidia/mL. Spore suspensions were prepared one day before inoculation and stored at 4 °C.

2.7. Cultures with/without B. subtilis

A yeast extract–sucrose (YES) broth medium was used for growth and aflatoxin production of *A. parasiticus* ATCC 15517 and *B. subtilis*. The medium was sterilized at 121 °C for 15 min and cooled to room temperature. Test tubes, each containing the same volume of the prepared medium, were inoculated with a spore suspension of *A. parasiticus* and inoculum of *B. subtilis* and then incubated at 28 °C for 9 days. An *A. parasiticus* culture grown in the absence of *B. subtilis* was used as control.

2.8. Determination of Fungal Growth and Aflatoxin Production

After incubation, mycelial mats from cultures were collected on dried, preweighed Whatman® qualitative filter paper, grade 1 (Whatman, Little Chalfont, Buckinghamshire, UK). They were then washed with distilled water and dried at 55–60 °C overnight. The dry weight of mycelial mats was used as the measurement of fungal growth.

Aflatoxin was extracted from the cultures and quantified using high-performance liquid chromatography (HPLC). The procedure used for extracting aflatoxin in this study was identical to that described previously [37]. The extract was evaporated to dryness under a stream of nitrogen gas, and trifluoroacetic acid (TFA) was added before redissolving the residue in an appropriate volume of injection solvent. TFA-treated standards and sample extracts were injected into the HPLC column.

The HPLC equipment comprised a Symmetry C_{18} column (15 cm by 3.9 mm (inner diameter)) and fluorescence detector (excitation at 365 nm and emission at 425 nm) of an Agilent 1200 Series HPLC System (Agilent Technologies, Inc., Santa Clara, CA, USA). The chromatograms were obtained with the mobile phase composed of water–acetonitrile (80:20, *v*/*v*).

2.9. Statistical Analysis

The data obtained from meju samples were compared using Student's *t*-test or analysis of variance. Significant differences among means were determined using Duncan's multiple range test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Changes in Bacillus Population

The population of aerobic mesophiles and *Bacillus* species in each of the meju samples during fermentation is shown in Figure 1. In meju samples, the population of aerobic mesophiles increased from $10^{5.12}$ cfu/g to $10^{10.56}$ cfu/g during 1 month of fermentation ($p < 0.05$), and after which a slight decline was observed. In the final product, the population of aerobic mesophiles was $10^{10.42}$ cfu/g. The aerobic mesophiles in fermenting meju were dominated by Gram-positive, catalase-positive rods, most bearing phase-bright spores. The dominant bacteria isolated from nutrient agar were assumed to belong to the genus *Bacillus*. The *Bacillus* species population in meju increased from $10^{4.03}$ cfu/g to $10^{8.56}$ cfu/g ($p < 0.05$). The highest increase in *Bacillus* population was observed during the first one month of fermentation, where the meju samples recorded a level of 10^8 cfu/g of *Bacillus*

population. The subsequent increases in their population during the next two and three months of fermentation were between 1×10^5 and 3×10^5.

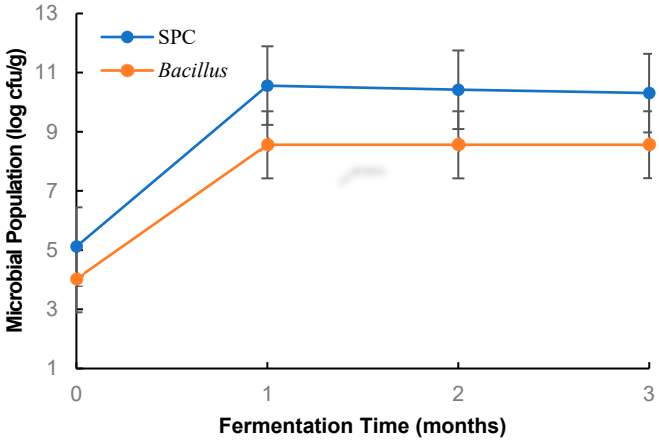

Figure 1. Changes in the population of aerobic mesophiles and *Bacillus* species during the fermentation of meju bricks. SPC: standard plate count.

3.2. Characterization and Identification of Bacillus Strain

A *Bacillus* strain was identified by its morphological characteristics and biochemical profile. Table 1 summarizes the results of the characterization of the isolated strain from meju. The strain was found to be an aerobic, Gram-positive, spore-forming rod, capable of moving, and to produce a mucoid colony when grown on LB agar plates. Further examination showed that most of these isolates from the meju produced acid from D-glucose, L-arabinose, D-xylose, and D-mannitol: hydrolyzed casein, gelatin, and starch; reduced nitrate, and grew at pH 5.7 and 6.0. Under microscopy, the morphology of the isolated strain was revealed as rod-shaped cells. Micrograph images indicated the strain had a long and straight rod shape (0.7–0.9 × 2.0–2.3 µm), as shown in Figure 2.

Table 1. Characteristics of *B. subtilis* K-0924 isolated from meju bricks.

Parameter of Characteristics	Results	Parameter of Characteristics	Results
Cell diameter > 1.0	-	Acids from	
Spores round	-	D-glucose	+
Endospore	+	L-arabinose	+
Gram stain	+	D-xylose	+
Form	Rod	D-mannitol	+
Sporangium swollen	-	Hydrolysis of	
Parasporal crystals	-	Casein	+
Catalase	+	Gelatin	+
Anaerobic growth	-	Starch	+
Voges–Proskauer test	+	Nitrate reduction	+
pH in V-P broth		Growth	
<6	+/-	5 °C	-
>7	-	20 °C	+
Gas from glucose	-	30 °C	+
Indole	+	50 °C	+
Acetyl methyl carbinol	+	60 °C	+
Citrate	-		

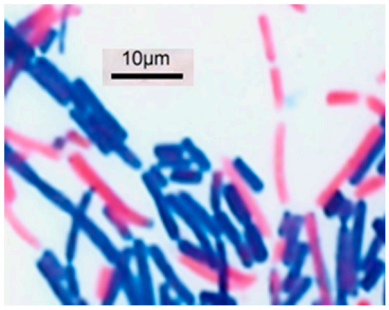

Figure 2. The *B. subtilis* K-0924 isolated from meju bricks. Result of Gram staining under microscope.

The biochemical characteristics of the strain were similar to *B. subtilis* using a *Bacillus* ID kit. Comparative 16S rDNA gene sequence analysis of the strain also showed that the strain was most closely related to *B. subtilis*, with sequence similarity of 99.5%. Based on the biochemical characteristics and phylogenetic distinctiveness, the strain was classified within the genus *Bacillus* as *B. subtilis*. We will refer to this newly isolated strain as K-0924.

3.3. Effect of B. subtilis K-0924 on the Growth of A. parasiticus and Aflatoxin Production

Growth of the *A. parasiticus* ATCC 15517 on YES broth was monitored using mycelial mats. Figure 3 shows the effect of *B. subtilis* on the growth of *A. parasiticus* (Figure 3). The growth of *A. parasiticus* increased over time on YES broth. A significant inhibition of fungal growth was observed from the third day when treated with *B. subtilis* ($p < 0.05$). When *A. parasiticus* was cultured alone on the YES broth, it produced dry weight of mycelia at the levels of 14.5–51.5 mg during the incubation period. However, only 2.6–11.6 mg of dry weight of mycelia was produced when *A. parasiticus* was combined with *B. subtilis*. On average, the mycelial growth was inhibited by 80.5% compared to the control cultures lacking *B. subtilis* ($p < 0.01$).

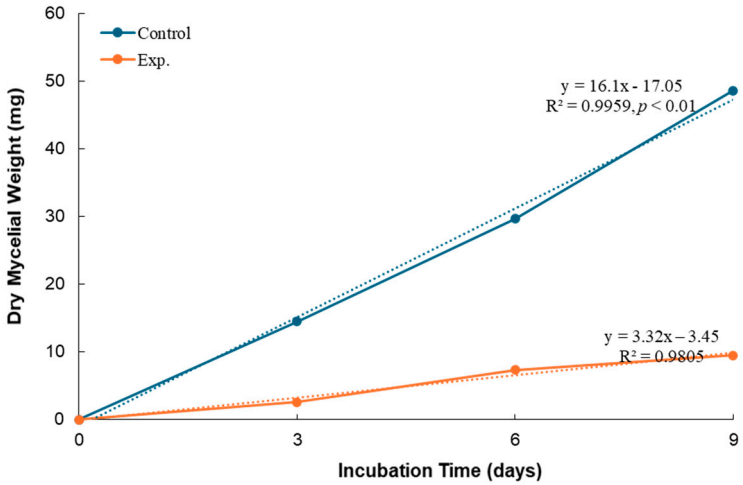

Figure 3. Effect of *B. subtilis* K-0924 on the growth of *A. parasiticus* ATCC 15517 in YES broth. Control: *A. parasiticus* was inoculated alone, Exp.: *A. parasiticus* was inoculated with *B. subtilis*.

Aflatoxin production was determined at the termination of incubation. The four aflatoxins were separated in the HPLC system, and all the peaks of aflatoxin B_1, B_2, G_1, and G_2 were eluted within 20 min (Figure 4). Figure 5 shows the effect of *B. subtilis* on the aflatoxin production by *A. parasiticus* (Figure 5). The production of aflatoxins on YES broth was also inhibited. There was a much lower level of aflatoxins when *A. parasiticus* was grown in the presence of *B. subtilis*. The total aflatoxin was reduced by 63.4% compared to the control cultures lacking *B. subtilis* ($p < 0.05$).

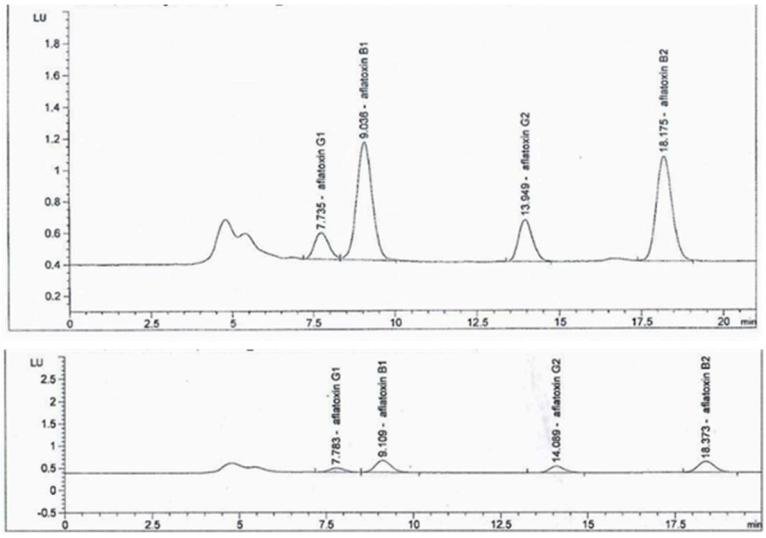

Figure 4. HPLC chromatograms of sample extract of aflatoxins. **Upper**; control, **lower**: exp.

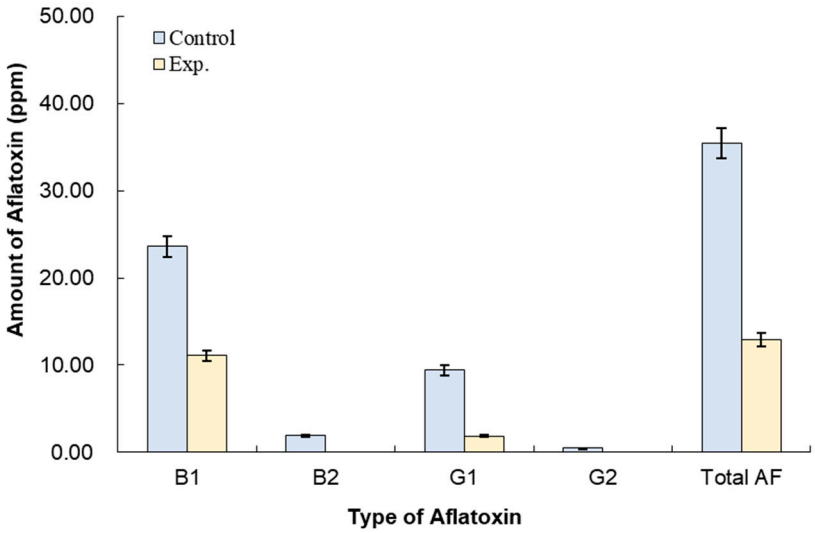

Figure 5. Effect of *B. subtilis* K-0924 on the aflatoxin production of *A. parasiticus* ATCC 15517 in YES broth. Control: *A. parasiticus* was inoculated alone, Exp.: *A. parasiticus* was inoculated with *B. subtilis*.

4. Discussion

The changes in aerobic mesophiles and *Bacillus* species in meju were evaluated during fermentation. The initial populations of aerobic mesophiles and *Bacillus* species in meju samples were 10^5 cfu/g and 10^4 cfu/g, respectively. The two microbial populations in meju increased over time, and the final counts exceeded 10^{10} c/fug and 10^8 cfu/g, respectively. The results of this work are similar to the findings of Choi et al. [7], who reported that the aerobic bacterial count in meju was 10^5 cfu/g at the initial stage and 10^{10} cfu/g after fermenting. Their results and the results of this study suggest that *Bacillus* species constitute the main share of the microbial population in meju, and they play a leading role in fermenting meju.

The isolated strain, *B. subtilis* K-0924, was found to be an aerobic, Gram-positive, motile, rod-shaped, and endospore-forming bacterium in the culture on LB medium at 30 °C. The morphological features of the strain were confirmed using a phase-contrast microscope. The growth temperature of the strain was investigated, and it grew between 20 °C and 60 °C, with optimal growth at 30–40 °C. The pH range for growth was measured, and the optimal pH values were 6–7. The carbohydrate fermentation test for D-glucose, L-arabinose,

D-xylose, and D-mannitol was active. The isolated strain was further characterized and confirmed based on 16S rDNA sequence analysis.

The results of this study are supported by several researchers who reported *Bacillus* species, mainly *B. subtilis*, as the predominant microorganisms in fermenting soybeans during meju production [38–40]. Amoa-Awua et al. [41] reported an increase in *Bacillus* population in soybean dawadawa, a condiment that was traditionally produced using the African locust bean during 24 h of fermentation, after which a slight incline was observed. However, *Bacillus* counts in the final product of fermented meju in this study were lower than the counts reported in soybean dawadawa by Ogbadu and Okagbue [42], Omafuvbe et al. [43], and Terlabie et al. [44]. In their results, 10^9 or 10^{10} to 10^{11} cfu/g of *Bacillus* was counted in the fermented soybean dawadawa, although the *Bacillus* populations in their samples at the initial stage were comparable with this study. The difference might be due to the nature of the strains themselves, substrate (type of beans), and/or fermentation condition.

The fermentation of soybeans into natto, thua nao, and kinema in Asian countries are also reported to be due to *B. subtilis* [30,45]. *B. subtilis* has also been reported to be responsible for the traditional alkaline fermentation of legumes and seeds in West Africa, including African locust bean seeds [41–44]. The isolation of *B. subtilis* from meju in this study is of significant importance, as it has been assumed to be one of the predominant species in meju, soy sauce, and soybean paste. However. the fermented meju sample was likely to contain more than one species of *Bacillus*.

Omafuvbe et al. [43] and Dakwa et al. [45] isolated other species, such as *B. licheniformis*, *B. pumilus*, *B. megaterium*, and *B. firmus*, from fermenting soybean dawadawa. Dakwa et al. [45] reported that *B. subtilis* accounted for 48–50% of the *Bacillus* population in soy dawadawa. Hence, further studies on the identification and characterization of the other *Bacillus* strains in meju should be conducted.

This study also investigated the inhibition of growth and aflatoxin production of *A. parasiticus* ATCC 15517 in the presence of *B. subtilis* K-0924 isolated from the meju we prepared in liquid culture. From the results presented, we can assume that parts of aflatoxins produced by *A. parasiticus* were degraded by the co-inoculation of *B. subtilis* K-0924. We could expect more inhibition by other bacteria associated with meju fermentation. Also, the metabolites produced by *B. subtilis* K-0924 that were responsible for degrading aflatoxins in the culture should be further investigated, and this is expected in future studies.

5. Conclusions

In this study, we investigated the inhibition effect of *B. subtilis* isolated from meju on the growth and aflatoxin production of *A. parasiticus*. Because both the bacterium and the mold are involved in meju fermentation, we undertook an overall prolonged process from meju preparation to observe the antifungal activity of the isolated strain from the meju. A *B. subtilis* K-0924 strain was identified based on morphological characteristics, biological profile, and genetic analysis using 16S rDNA sequence analysis. The effect of the *B. subtilis* K-0924 strain on *A. parasiticus* ATCC 15517 was evaluated in two ways: mycelial growth and production of four types of aflatoxins. We found that *B. subtilis* K-0924 has inhibitory effects on both mold growth and aflatoxin production. Although its effect on aflatoxin production was less pronounced than growth retardation, we anticipate more inhibition by other bacteria associated with fermentation in meju. From the results, we can suggest that *B. subtilis* K-0924 has potential application as a probiotic.

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Conflicts of Interest: The authors declare no conflicts of interest.

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