


## Biocontrol Activity of Volatile-Producing *Bacillus megaterium* and *Pseudomonas protegens* Against *Aspergillus* and *Penicillium* spp. Predominant in Stored Rice Grains: Study II

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### ABSTRACT

In our previous studies, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15 have been shown to be antagonistic to *Aspergillus flavus* in stored rice grains. In this study, the biocontrol activities of these strains were evaluated against *Aspergillus candidus*, *Aspergillus fumigatus*, *Penicillium fellutanum*, and *Penicillium islandicum*, which are predominant in stored rice grains. *In vitro* and *in vivo* antifungal activities of the bacterial strains were evaluated against the fungi on media and rice grains, respectively. The antifungal activities of the volatiles produced by the strains against fungal development and population were also tested using I-plates. In *in vitro* tests, the strains produced secondary metabolites capable of reducing conidial germination, germ-tube elongation, and mycelial growth of all the tested fungi. In *in vivo* tests, the strains significantly inhibited the fungal growth in rice grains. Additionally, in I-plate tests, strains KU143 and AS15 produced volatiles that significantly inhibited not only mycelial growth, sporulation, and conidial germination of the fungi on media but also fungal populations on rice grains. GC-MS analysis of the volatiles by strains KU143 and AS15 identified 12 and 17 compounds, respectively. Among these, the antifungal compound, 5-methyl-2-phenyl-1H-indole, was produced by strain KU143 and the antimicrobial compounds, 2-butyl 1-octanal, dimethyl disulfide, 2-isopropyl-5-methyl-1-heptanol, and 4-trifluoroacetoxyhexadecane, were produced by strain AS15. These results suggest that the tested strains producing extracellular metabolites and/or volatiles may have a broad spectrum of antifungal activities against the grain fungi. In particular, *B. megaterium* KU143 and *P. protegens* AS15 may be potential biocontrol agents against *Aspergillus* and *Penicillium* spp. during rice grain storage.

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## 1. Introduction


Stored rice grains contain various microbes such as bacteria and fungi, in which the microbial structure depends on not only the harvesting process but also the field and storage conditions. Deleterious fungal growth in stored rice grains leads to reduced milling quality and nutritional values, discoloration, and production of hazardous mycotoxins [1]. During grain storage, low relative humidity promotes the growth of xerophilic fungi (i.e., dry condition-tolerant fungi) such as *Aspergillus* and *Penicillium* spp., which are predominant in rice grains [2–4]. These fungi, along with *Fusarium* spp., are responsible for mycotoxin contamination of rice grains [5]. Mycotoxins, as secondary metabolites of certain filamentous fungi, are known to elicit toxic biological responses in humans and animals [4,6].

*Aspergillus flavus* is the most deleterious fungus in stored rice grains, and it receives particular

attention because of its ability to produce potent carcinogenic aflatoxins [7]. Along with *A. flavus*, *Aspergillus candidus*, *Aspergillus fumigatus*, *Penicillium fellutanum*, and *Penicillium islandicum* are other major contaminants of stored rice grains [2,8–10]. First of all, *A. candidus* is a common fungus of the natural mycoflora in rice grains [11]. This fungus was reported to represent a potential respiratory hazard and a strong immune-stimulating factor for grain workers [12]. Moreover, *A. candidus* produces several phenolic compounds such as cytotoxic terphenyllin and xanthoascin (a xanthocillin analog), which are hepato- and cardio-toxic to animals [13,14]. *A. fumigatus* is the main causative agent of invasive aspergillosis, a life-threatening disease caused by inhaled conidia, particularly in immunocompromised individuals [15]. Several ergot alkaloids were found to be associated with *A. fumigatus* conidia in a respirable form at high levels. Ergot

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alkaloids are known to cause several health problems in humans and animals, including negative effects on the cardiovascular, nervous, and immune systems [16]. In addition, *P. islandicum*, the main cause of “yellow rice” in Japan, can produce the toxic peptide, islanditoxin [17]. *P. fellutanum* is also a myco-toxigenic fungus producing diketopiperazine alkaloids (fellutanines A–E), in addition to other alkaloids (agroclavine I and epoxyagroclavine I) [18,19]. Because of the toxic activities and health hazards associated with the fungal contamination of stored rice grains, several attempts have been made in search of environmentally safe, efficient, and sustainable control methods [4,6,20–22].

Conventionally, synthetic agro-chemicals have been used for the management of seed-borne fungal diseases. Fungicides such as ammonia, benomyl, formaldehyde, iprodione, propionic and sorbic acid, and thiabendazole have been applied for controlling grain fungi during storage [23–28]. However, fungicide application on stored grains has been limited because of their adverse effects on the sensory quality of grains, inefficiency in storage conditions, and their toxic potential [29]. Moreover, increasing evidence and awareness of related health issues, as well as the occurrence of fungicide resistance, has highlighted the need for environmentally sound and efficient alternatives to chemical application [29]. A promising approach for controlling deleterious fungi in stored grains may be the use of antagonistic microbes. In particular, microbes producing antifungal volatiles, which is one of the most significant biocontrol traits, may have a potential to control deleterious grain fungi [30,31]. For example, the volatile compound, 2-phenylethanol, produced by the biocontrol yeast *Pichia anomala* suppressed *A. flavus* growth and aflatoxin production [32]. Additionally, antagonistic microbes can also serve as an untapped resource of bioactive metabolites [6,33].

In our previous study, three antagonistic bacterial strains, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15, were selected from 460 strains isolated from stored rice grains in Korea [20,34]. These strains exhibited significant biocontrol activities against *A. flavus* and aflatoxin production in stored rice grains [20]. Moreover, volatiles from *B. megaterium* KU143 and *P. protegens* AS15 were found to inhibit *A. flavus* growth and aflatoxin production [21]. Further examination of antifungal activities of the antagonistic strains against grain fungi such as *A. candidus*, *A. fumigatus*, *P. fellutanum*, and *P. islandicum* are needed to enable these strains to use as biocontrol agents during grain storage. Therefore, the objectives of this study were (1) to evaluate the antifungal activities of the bacterial strains against *Aspergillus* and *Penicillium* spp. predominant in stored rice

grains, (2) to investigate the antifungal activities of the volatiles produced by the strains against the tested fungi, and (3) to identify bacterial volatile compounds using gas chromatography-mass spectrometry (GC-MS) analysis.

## 2. Materials and methods

### 2.1. Fungal and bacterial strains and rice grains

The antagonistic bacterial strains, *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15, were stored in nutrient broth (NB) containing 20% glycerol at  $-70^{\circ}\text{C}$  until used. To prepare bacterial cultures, the strains were streaked on nutrient agar (NA) and incubated at  $28^{\circ}\text{C}$  for 2 d. Single colonies were transferred to NB and incubated in a rotary shaker (160 rpm) at  $28^{\circ}\text{C}$  for 2 d. The cultured bacterial cells were harvested and washed twice by centrifugation at  $5000\times g$  for 15 min, and suspended in a 10 mM  $\text{MgSO}_4$  solution. The bacterial suspensions were adjusted to  $10^8$  cells/ml ( $\text{OD}_{600} = 0.5$ ) with the  $\text{MgSO}_4$  solution, using a spectrophotometer.

The tested fungi, *A. candidus* AC317, *A. fumigatus* AF8, *P. fellutanum* KU53, and *P. islandicum* KU101, isolated from stored rice grains in Korea [8–10], were stored on potato dextrose agar (PDA) at  $4^{\circ}\text{C}$  until used. To prepare fungal inocula, the isolates were cultured on PDA for 5 d at  $28^{\circ}\text{C}$ . Subsequently, conidia were harvested using sterile distilled water (SDW) containing 0.03% Tween 20. Harvested conidia were adjusted to  $10^7$  conidia/ml using a hemocytometer. Unhulled, stored rice grains (cv. “Ilpum”) from the Korea University Farm (Namyangju, Korea) were surface-sterilized using 1% sodium hypochlorite for 3 min and 70% ethanol for 5 min [35]; then, washed with SDW three times and blotted on sterile Whatman (No. 1) filter papers.

### 2.2. In vitro antifungal activity of antagonistic bacterial strains and bacterial culture filtrates in media

Antifungal activity of antagonistic bacterial strains was examined against the tested grain fungi, *A. candidus* AC317, *A. fumigatus* AF8, *P. fellutanum* KU53, and *P. islandicum* KU101, in a dual-culture assay. The assay was based on the inhibition of the fungal mycelia on PDA plates (90 mm in diameter), streaked in the center with each tested bacterial strain, *Sphingomonas aquatilis* KU408 (bacterial negative control), or SDW (untreated control). Streaked plates were inoculated with a conidial suspension (2  $\mu\text{l}$ ) of each tested fungus, prepared as described above, to the opposite edges of the plates.

The plates were sealed with Parafilm M (Sigma-Aldrich, St. Louis, MO), and incubated at 28 °C until mycelia in the untreated controls reached the center of the plates. Inhibition (mm) of mycelial growth of the tested fungi was determined and compared with the mycelial growth of the untreated control plates.

Culture filtrates of antagonistic strains were examined for their activity against conidial germination, germ-tube elongation, and mycelial growth of the tested fungi. The cell-free NB culture filtrates of the bacterial strains, prepared as described above, were obtained using 0.22 µm micro-filters (Merck Millipore, Darmstadt, Germany). One milliliter of the culture filtrate of each tested strain, *S. aquatilis* KU408 (bacterial negative control) or NB (untreated control) was added to 9 ml of potato dextrose broth (PDB) containing 10<sup>5</sup> conidia/ml. These samples were incubated at 28 °C for 10 h and conidial germination percentage was assessed by counting the number of germinated conidia among a total of 100 conidia observed. Subsequently, germ-tube lengths (µm) were measured using 20 germinated conidia. Another set of samples was incubated at 28 °C for 2 d in a rotary shaker (200 rpm) for assessing fungal growth. Mycelia of the tested fungi were harvested by filtration using Whatman (No. 1) filter papers, and mycelial dry weights (mg) were determined after drying the mycelia at 60 °C for 2 d.

### 2.3. In vivo antifungal activity of antagonistic bacterial strains in rice grains

Biocontrol activity of antagonistic bacterial strains against the tested fungi was evaluated according to the procedure described by Mannaa et al. [20]. The surface-sterilized rice grains (2 g) were treated with the bacterial suspensions for 3 h. *S. aquatilis* KU408, 10 mM MgSO<sub>4</sub> solution, and SDW were used as negative controls and a commercial fungicide, Benlate<sup>®</sup> (50% benomyl; Syngenta Korea, Seoul, Korea) at a concentration of 3 mg/g of rice grains was used as a positive control. Treated rice grains were inoculated with 200 µl of 10<sup>7</sup> conidia/ml, equivalent to 10<sup>6</sup> conidia/g dry weights of rice grains.

After 7 d of inoculation, fungal and bacterial populations of the treated rice grains were evaluated as followings. Rice grains were finely ground aseptically with an analytical mill (IKA A11 basic, IKA Works, Wilmington, NC). Grains (1 g) were suspended in 10 ml SDW and incubated in a rotary shaker (160 rpm) at 28 °C for 30 min. After incubation, rice suspensions were serially diluted 10-fold, and 200 µl of each dilution was smeared on 18% glycerol agar (DG18; Fluka 40587, Sigma-Aldrich, St. Louis, MO), followed by incubation at 28 °C for 4 d for the assessment of fungal populations [36]. Another 200 µl

of each dilution was smeared on NA supplemented with cycloheximide (50 mg/l) and incubated at 28 °C for 2 d to assess bacterial populations. Fungal and bacterial populations were expressed as colony-forming units (CFUs)/g dry weights of rice grains.

### 2.4. In vitro antifungal activity of bacterial volatiles on I-plates

*In vitro* antifungal activity of the volatiles produced by antagonistic bacterial strains against mycelial growth, sporulation, and conidial germination of the tested fungi was conducted using I-plates (plates containing a center partition; Fisher Scientific, Pittsburgh, PA) according to the method described by Mannaa et al. [21]. Briefly, 10 ml of NA supplemented with 50 mg/l cycloheximide was applied to one side of the I-plate, and PDA was poured on the opposite side. Then, 100 µl of the bacterial suspension of each tested strain, *S. aquatilis* KU408 (bacterial negative control), or 10 mM MgSO<sub>4</sub> solution (untreated control) was smeared onto the NA side of the I-plate. After 24 h of incubation at 28 °C, 2 µl of the conidial suspension (10<sup>7</sup> conidia/ml) of each tested fungus was drop-inoculated on the opposite (containing PDA) edges of the I-plates. These plates were sealed with Parafilm and incubated at 28 °C until mycelia approximately reached the center of the untreated control plates. Mycelial growth area (cm<sup>2</sup>) was determined using ImageJ software (NIH Image J system, Bethesda, MD) after photography [37]. To evaluate fungal sporulation, conidia in the plates were harvested with SDW containing 0.03% Tween 20, vortexed for 1 min, and the number of conidia was counted using a hemocytometer. Fungal sporulation was expressed as the total number of conidia from each plate, divided by the mycelial growth area (cm<sup>-2</sup>) of each plate. The conidial germination percentage of the tested fungi was then determined using the harvested conidia as described above. The harvested conidia were filtered through two layers of sterile cheesecloth and transferred to PDB at a final concentration of 10<sup>5</sup> conidia/ml. After stationary incubation of the samples (three sub-replicates per sample) at 28 °C for 10 h, the number of germinated conidia was counted among a total of 100 conidia observed per sample.

### 2.5. In vivo antifungal activity of bacterial volatiles on I-plates

*In vivo* antifungal activity of the volatiles produced by antagonistic bacterial strains against the tested fungi was examined on unhulled rice grains on I-plates. A 100 µl aliquot of the bacterial suspension of each antagonistic strain, the negative control

strain or 10 mM MgSO<sub>4</sub> solution was smeared on I-plates containing NA as described above, and incubated at 28 °C. After 24 h of incubation, surface-sterilized grains (2 g) were placed on the opposite side of the I-plate, and inoculated with 200 µl of the conidial suspension of each tested fungus, as described above. These plates were sealed with Parafilm and incubated at 28 °C. After 7 d of incubation, the populations of the tested fungi in grains were evaluated as described above, and expressed as CFU/g dry weights of rice grains.

### 2.6. GC-MS analysis of bacterial volatiles

To identify the volatile compounds produced by *B. megaterium* KU143 and *P. protegens* AS15, GC-MS analysis was conducted using the method described by Sang et al. [30]. Bacterial strains were streaked and grown on tryptic soy agar (TSA) and single colonies were pre-cultured in 2 ml of tryptic soy broth (TSB) as described for the bacterial preparation. These pre-cultured strains were further cultured in 1000 ml baffled Erlenmeyer flasks containing 100 ml of TSB. That is, flasks (three flasks per treatment) containing each tested strain or TSB (control) were sealed with two layers of Parafilm and incubated in a rotary shaker (160 rpm) at 28 °C. After 24 h of incubation, volatiles from the headspace of each flask were collected using a 50 ml gastight syringe (Hamilton Co., Reno, NV). Then, 10 ml of the collected volatiles was eluted in 500 µl of hexane and 2 µl of this solution was injected into a GC-MS system (Agilent 7890A for GC and 5975C MSD for MS; Agilent Technologies, Santa Clara, CA) equipped with a DB-5 MS column (307 m × 0.25 mm × 0.25 µm) (Agilent Technologies, Santa Clara, CA). The running conditions were: injector at 200 °C; detector at 210 °C; column oven at 30 °C for 3 min with programming at a rate of 10 °C/min up to 180 °C, then ramped up at a rate of 40 °C min<sup>-1</sup> to 200 °C for 2 min. Helium was the carrier gas at a flow rate of 1 ml/min. The mass spectra of the obtained peaks were compared with the spectra corresponding to the best library match using Chemstation software (Hewlett Packard, Palo Alto, CA).

### 2.7. Statistical analysis

The experiments were designed to be completely randomized designs and were conducted twice with three replicates per treatment. Data from repeated experiments were combined after confirming the homogeneity of variances using Levene's test [38]. For analysis of fungal populations, the data were analyzed after log transformation. Analysis of variance (ANOVA) was conducted using the general

linear model (GLM) procedures, and the means were separated using the least significant difference (LSD) test at  $p < .05$ . Statistical analysis of the data was performed using the Statistical Analysis Systems software (SAS Institute, Cary, NC).

## 3. Results

### 3.1. In vitro antifungal activity of antagonistic bacterial strains and bacterial culture filtrates against *Aspergillus* and *Penicillium* in media

When the antifungal activity of antagonistic bacterial strains was tested in a dual-culture assay, all the tested strains significantly ( $p < .05$ ) inhibited mycelial growth of the tested fungi compared with the bacterial negative control strain, *S. aquatilis* KU408 (Table 1). Among the tested strains, *P. protegens* AS15 caused the most significant ( $p < .05$ ) reduction in mycelial growth in all the tested fungi, followed by *B. megaterium* KU143 (Table 1).

Similarly, the cell-free culture filtrates of the antagonistic strains significantly ( $p < .05$ ) inhibited conidial germination, germ-tube elongation, and mycelial dry weights of all the tested fungi, compared with untreated and negative controls (Table 1). *S. aquatilis* KU408 showed no antifungal activity against the tested fungi as detected in the untreated control. As observed in the dual-culture assays, *P. protegens* AS15 showed the highest antifungal activity against all the tested fungi (Table 1).

### 3.2. In vivo antifungal activity of antagonistic bacterial strains against *Aspergillus* and *Penicillium* in rice grains

When biocontrol activity of the antagonistic bacterial strains was examined against the grain fungi on unhulled rice grains, *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15 significantly ( $p < .05$ ) inhibited populations of all the tested fungi when compared with SDW and MgSO<sub>4</sub> (untreated controls) treatments (Supplementary Figure 1; Figure 1). Among these strains, *P. protegens* AS15 caused the largest reduction in fungal populations in the grains, regardless of the tested fungus. As expected, the commercial agrochemical Benlate® (positive control) caused the largest population reduction in the tested fungi among all the treatments. However, *S. aquatilis* KU408 (bacterial negative control), failed to reduce the fungal populations compared with the untreated controls in the tested fungi (Figure 1).

The tested bacterial strains significantly ( $p < .05$ ) enhanced the bacterial populations in grains compared with the untreated controls, regardless of the tested fungus (Figure 1). *S. aquatilis* KU408 also

**Table 1.** Antifungal activities of antagonistic bacterial strains, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15, and negative control strain, *Sphingomonas aquatilis* KU408, on mycelial growth of *Aspergillus candidus* AC317, *Aspergillus fumigatus* AF8, *Penicillium fellutanum* KU53, and *Penicillium islandicum* KU101 in dual-culture assays and the effects of cell-free culture filtrates of these strains on conidial germination, germ-tube lengths, and mycelial dry weights of the fungi in potato-dextrose broth tests.

Treatment	Mycelial growth inhibition (mm)		Conidial germination (%)		Germ tube length ( $\mu$ m)		Mycelial dry weight (mg)	
	<i>A. candidus</i>	<i>A. fumigatus</i>	<i>A. candidus</i>	<i>A. fumigatus</i>	<i>A. candidus</i>	<i>A. fumigatus</i>	<i>A. candidus</i>	<i>A. fumigatus</i>
Control <sup>a</sup>	— <sup>b</sup>	—	33.2 $\pm$ 7.1 A <sup>c</sup>	78.2 $\pm$ 4.1 A	39.4 $\pm$ 3.9 A	51.5 $\pm$ 4.2 A	31.3 $\pm$ 8.8 A	64.7 $\pm$ 8.1 A
KU408	1.0 $\pm$ 1.0 D	0.1 $\pm$ 0.4 D	30.9 $\pm$ 4.9 AB	71.9 $\pm$ 3.1 A	37.9 $\pm$ 3.6 A	50.9 $\pm$ 5.5 A	32.0 $\pm$ 8.3 A	62.3 $\pm$ 8.9 A
KU313	6.0 $\pm$ 2.0 C	6.5 $\pm$ 1.8 C	25.3 $\pm$ 5.0 B	52.3 $\pm$ 3.7 B	21.3 $\pm$ 3.0 B	27.0 $\pm$ 2.6 B	22.3 $\pm$ 5.3 B	45.0 $\pm$ 4.0 B
KU143	12.7 $\pm$ 2.6 B	9.9 $\pm$ 2.6 B	19.2 $\pm$ 2.0 C	37.0 $\pm$ 3.2 C	17.8 $\pm$ 1.9 C	22.6 $\pm$ 3.7 C	21.7 $\pm$ 3.7 B	38.0 $\pm$ 4.4 B
AS15	17.2 $\pm$ 1.4 A	14.2 $\pm$ 3.8 A	12.3 $\pm$ 2.9 D	25.7 $\pm$ 2.2 C	17.1 $\pm$ 3.5 C	18.7 $\pm$ 4.4 C	20.2 $\pm$ 6.9 B	29.2 $\pm$ 3.1 C

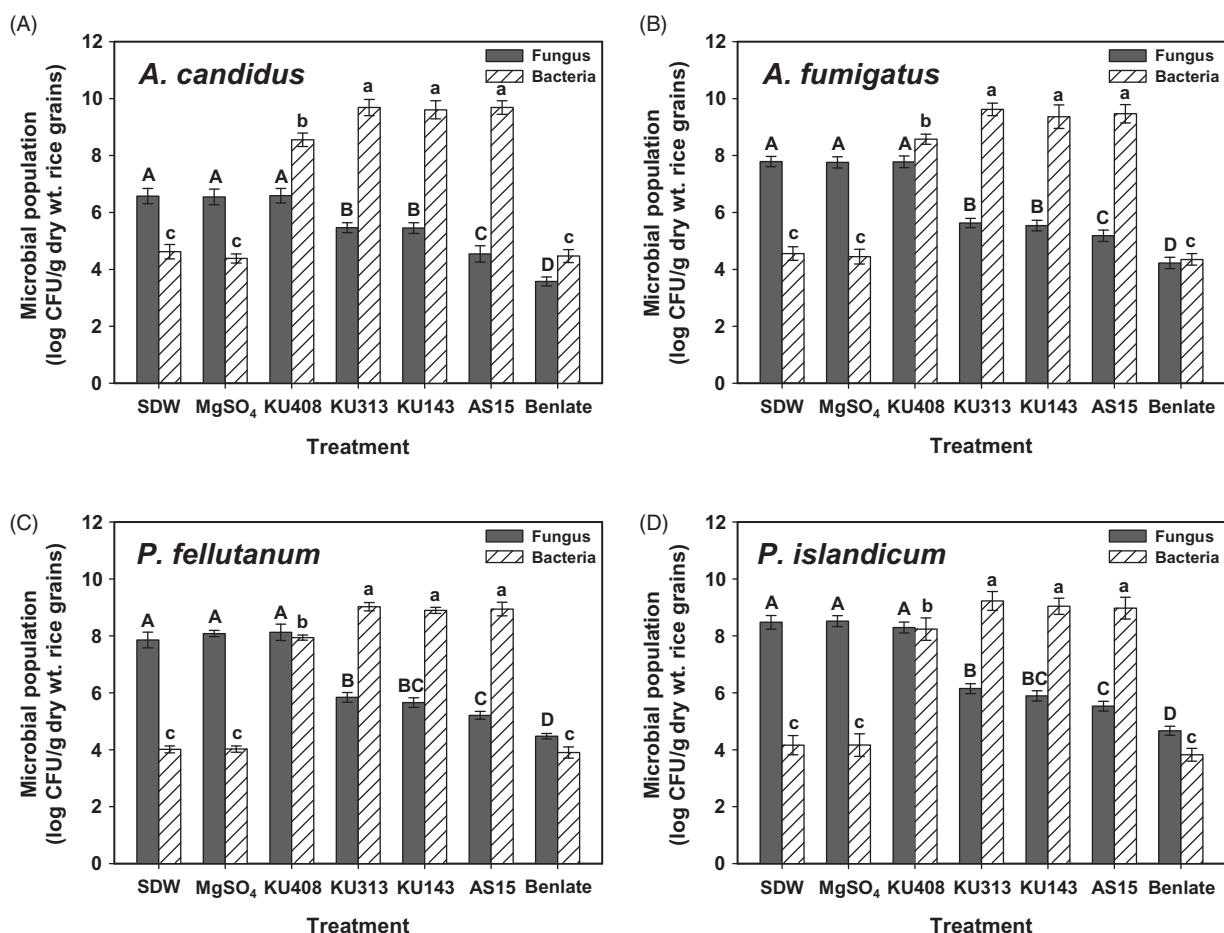
  

Treatment	Mycelial growth inhibition (mm)		Conidial germination (%)		Germ tube length ( $\mu$ m)		Mycelial dry weight (mg)	
	<i>P. fellutanum</i>	<i>P. islandicum</i>	<i>P. fellutanum</i>	<i>P. islandicum</i>	<i>P. fellutanum</i>	<i>P. islandicum</i>	<i>P. fellutanum</i>	<i>P. islandicum</i>
Control	—	—	38.6 $\pm$ 3.6 A	35.9 $\pm$ 2.3 A	75.4 $\pm$ 2.8 A	63.2 $\pm$ 10.8 A	59.0 $\pm$ 4.8 A	45.0 $\pm$ 4.9 A
KU408	1.7 $\pm$ 1.1 D	1.6 $\pm$ 1.5 D	38.2 $\pm$ 3.4 A	34.4 $\pm$ 5.2 A	76.4 $\pm$ 3.9 A	68.2 $\pm$ 10.1 A	55.7 $\pm$ 5.3 A	42.2 $\pm$ 3.0 A
KU313	6.4 $\pm$ 1.5 C	7.5 $\pm$ 1.8 C	27.8 $\pm$ 5.7 B	24.2 $\pm$ 4.7 B	43.2 $\pm$ 7.9 B	32.4 $\pm$ 2.8 B	34.3 $\pm$ 4.7 B	22.5 $\pm$ 3.8 B
KU143	12.4 $\pm$ 2.0 B	12.6 $\pm$ 1.4 B	16.6 $\pm$ 3.4 C	17.2 $\pm$ 3.7 C	30.3 $\pm$ 3.4 C	30.6 $\pm$ 2.3 B	25.0 $\pm$ 2.2 C	15.5 $\pm$ 2.8 C
AS15	17.6 $\pm$ 1.1 A	17.3 $\pm$ 1.2 A	9.0 $\pm$ 1.9 D	12.1 $\pm$ 2.5 D	27.2 $\pm$ 3.2 C	26.3 $\pm$ 1.5 B	15.2 $\pm$ 2.3 D	8.0 $\pm$ 1.4 D

<sup>a</sup>The controls were sterile distilled water in dual-culture assays and sterile nutrient broth in potato-dextrose broth tests.

<sup>b</sup>Not detected.

<sup>c</sup>Means  $\pm$  standard deviations ( $n = 6$ ) with different capital letters in each column are significantly ( $p < .05$ ) different between treatments according to the least significant difference test.



**Figure 1.** Antifungal activities and populations of antagonistic bacterial strains, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15, and negative control strain *Sphingomonas aquatilis* KU408 against (A) *Aspergillus candidus* AC317, (B) *Aspergillus fumigatus* AF8, (C) *Penicillium fellutanum* KU53, and (D) *Penicillium islandicum* KU101, 7 d after treatment in unhulled rice grains. Sterile distilled water (SDW) and 10 mM MgSO<sub>4</sub> solution were used as negative controls whereas a commercial fungicide, Benlate<sup>®</sup> was used as a positive control. Fungal populations were determined after 4 d of incubation at 28 °C on DG18 and total bacterial populations were evaluated after 2 d of incubation at 28 °C on nutrient agar. Different letters on the vertical bars with error bars (standard deviations,  $n = 6$ ) indicate significant differences between treatments according to the least significant difference test at  $p < .05$ . CFU: colony-forming unit.

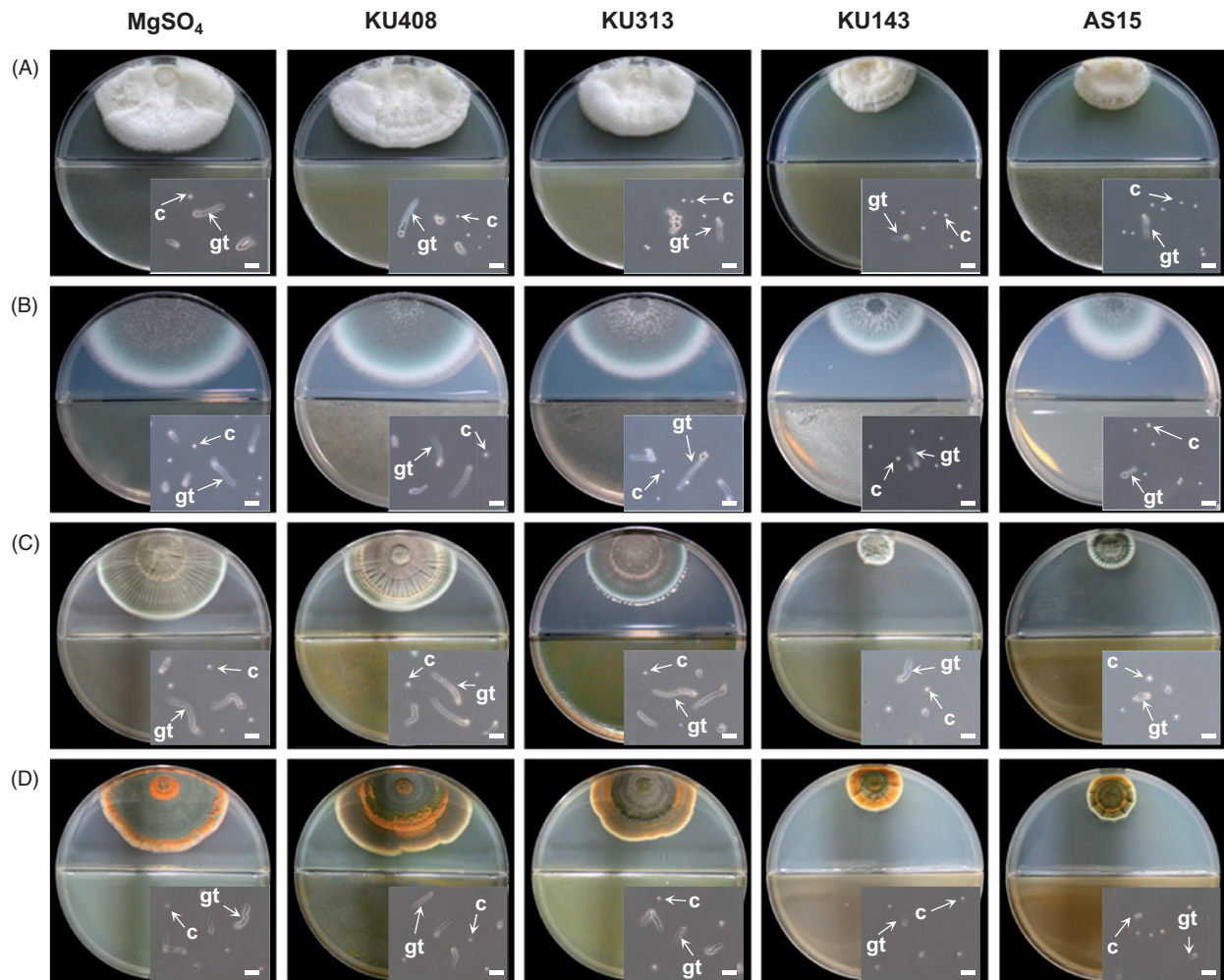
showed larger bacterial populations than the untreated controls for all the tested fungi. However, the tested antagonistic strains exhibited significantly ( $p < .05$ ) higher bacterial populations than the negative control strain. Untreated and positive control treatments showed similar levels of bacterial populations in the grains for all the tested fungi (Figure 1).

### 3.3. In vitro antifungal activity of bacterial volatiles against *Aspergillus* and *Penicillium* on I-plates

Volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly ( $p < .05$ ) inhibited the mycelial growth of all the tested fungi compared with *M. testaceum* KU313, *S. aquatilis* KU408 (bacterial negative control), and  $MgSO_4$  (untreated control) treatments (Figure 2 and Table 2). In addition, volatiles from *M. testaceum* KU313 significantly ( $p < .05$ ) reduced the mycelial growth

of *A. fumigatus* AF8 and *P. islandicum* KU101, compared with the negative and untreated controls. The volatiles also significantly ( $p < .05$ ) reduced the mycelial growth of *A. candidus* AC317 and *P. fellutanum* KU53 compared with the untreated control, though these were not significant compared with the bacterial negative control. As expected, *S. aquatilis* KU408 failed to inhibit the mycelial growth of the tested fungi (Figure 2 and Table 2).

In the case of bacterial volatile activity against fungal sporulation, volatiles from *B. megaterium* KU143 and *P. protegens* AS15 significantly ( $p < .05$ ) inhibited fungal sporulation regardless of the tested fungus (except for the inhibition of *A. candidus* by strain AS15) compared with *M. testaceum* and negative and untreated controls (Table 2). Volatiles from *M. testaceum* KU313 did not inhibit the sporulation of the tested fungi except for *A. fumigatus* AF8 compared with negative and untreated controls.



**Figure 2.** Photographs of antifungal activities of the volatiles produced by antagonistic bacterial strains, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15, and negative control strain *Sphingomonas aquatilis* KU408 against (A) *Aspergillus candidus* AC317, (B) *Aspergillus fumigatus* AF8, (C) *Penicillium fellutanum* KU53, and (D) *Penicillium islandicum* KU101 on the I-plates. Bacterial strains or 10 mM  $MgSO_4$  solution (untreated control) were smeared on one side (nutrient agar) of the I-plates and the other side (potato dextrose agar) was inoculated with a conidial suspension of the tested fungi 24 h after the bacterial treatment. Insets are photographs of the conidial germination of the fungi affected by the bacterial volatiles or  $MgSO_4$  solution. C: conidium; gt: germ tube. Scale bar, 20  $\mu$ m.

**Table 2.** Antifungal activities of the volatiles produced by antagonistic bacterial strains, *Bacillus megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15, and negative control strain, *Sphingomonas aquatilis* KU408, on mycelial growth, sporulation, and conidial germination of *Aspergillus candidus* AC317, *Aspergillus fumigatus* AF8, *Penicillium fellutanum* KU53, and *Penicillium islandicum* KU101 on I-plates.

Treatment	Mycelial growth (cm <sup>2</sup> )		Sporulation (log no. conidia cm <sup>-2</sup> mycelia)		Conidial germination (%)	
	<i>A. candidus</i>	<i>A. fumigatus</i>	<i>A. candidus</i>	<i>A. fumigatus</i>	<i>A. candidus</i>	<i>A. fumigatus</i>
MgSO <sub>4</sub>	14.3 ± 1.5 A <sup>a</sup>	17.3 ± 2.1 A	7.4 ± 0.1 A	8.1 ± 0.1 A	23.3 ± 3.6 A	64.2 ± 3.5 A
KU408	13.6 ± 1.7 AB	17.8 ± 1.7 A	7.3 ± 0.1 AB	8.0 ± 0.1 A	22.0 ± 3.3 A	63.5 ± 3.0 A
KU313	10.5 ± 1.4 B	15.1 ± 1.6 B	7.3 ± 0.2 AB	7.9 ± 0.1 B	15.0 ± 2.0 AB	49.3 ± 3.4 B
KU143	6.6 ± 0.3 C	8.5 ± 1.1 C	7.1 ± 0.1 C	6.5 ± 0.2 D	5.5 ± 0.9 C	9.2 ± 1.0 C
AS15	6.1 ± 0.3 C	7.9 ± 0.7 C	7.3 ± 0.2 B	7.4 ± 0.2 C	6.5 ± 0.8 BC	12.5 ± 1.4 C

Treatment	Mycelial growth (cm <sup>2</sup> )		Sporulation (log no. conidia cm <sup>-2</sup> mycelia)		Conidial germination (%)	
	<i>P. fellutanum</i>	<i>P. islandicum</i>	<i>P. fellutanum</i>	<i>P. islandicum</i>	<i>P. fellutanum</i>	<i>P. islandicum</i>
MgSO <sub>4</sub>	14.1 ± 1.4 A	14.5 ± 0.7 A	8.7 ± 0.1 A	8.5 ± 0.3 A	37.8 ± 1.2 A	36.7 ± 2.2 A
KU408	12.9 ± 1.5 AB	15.0 ± 1.1 A	8.7 ± 0.2 A	8.2 ± 0.3 A	38.2 ± 3.4 A	35.0 ± 3.4 A
KU313	11.8 ± 1.4 B	12.9 ± 1.6 B	8.4 ± 0.3 A	8.2 ± 0.3 A	37.0 ± 3.0 A	34.8 ± 3.2 A
KU143	2.0 ± 0.3 C	3.3 ± 0.2 C	7.0 ± 0.2 B	6.4 ± 0.3 B	7.8 ± 0.9 B	11.0 ± 1.6 B
AS15	2.7 ± 0.3 C	3.2 ± 0.2 C	7.0 ± 0.4 B	6.3 ± 0.3 B	11.3 ± 1.3 B	13.7 ± 2.0 B

<sup>a</sup>Means ± standard deviations ( $n=6$ ) with different letters in each column are significantly ( $p < .05$ ) different between treatments according to the least significant difference test.

*S. aquatilis* KU408 did not inhibit the fungal sporulation (Table 2).

In the tests of bacterial volatile effects on conidial germination, volatiles from *B. megaterium* KU143 and *P. protegens* AS15 significantly ( $p < .05$ ) inhibited the conidial germination of the tested fungi (except the inhibition of *A. candidus* by strain AS15) compared with *M. testaceum* KU313, and *S. aquatilis* KU408, and MgSO<sub>4</sub> treatments (Figure 2 and Table 2). Volatiles from *M. testaceum* KU313 significantly ( $p < .05$ ) reduced the germination of *A. fumigatus* AF8 only compared with untreated and negative controls (Figure 2 and Table 2).

### 3.4. In vivo antifungal activity of bacterial volatiles against *Aspergillus* and *Penicillium* on I-plates

When the antifungal activity of bacterial volatiles was evaluated against the populations of the tested fungi using unhulled rice grains, volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 significantly ( $p < .05$ ) inhibited the fungal populations, compared with *M. testaceum* KU313, *S. aquatilis* KU408 (bacterial negative control), and MgSO<sub>4</sub> (untreated control) treatments, regardless of the tested fungus (Supplementary Figure 2; Figure 3). Volatiles from *M. testaceum* failed to reduce the populations of the tested fungi, compared with negative and untreated controls (Figure 3).

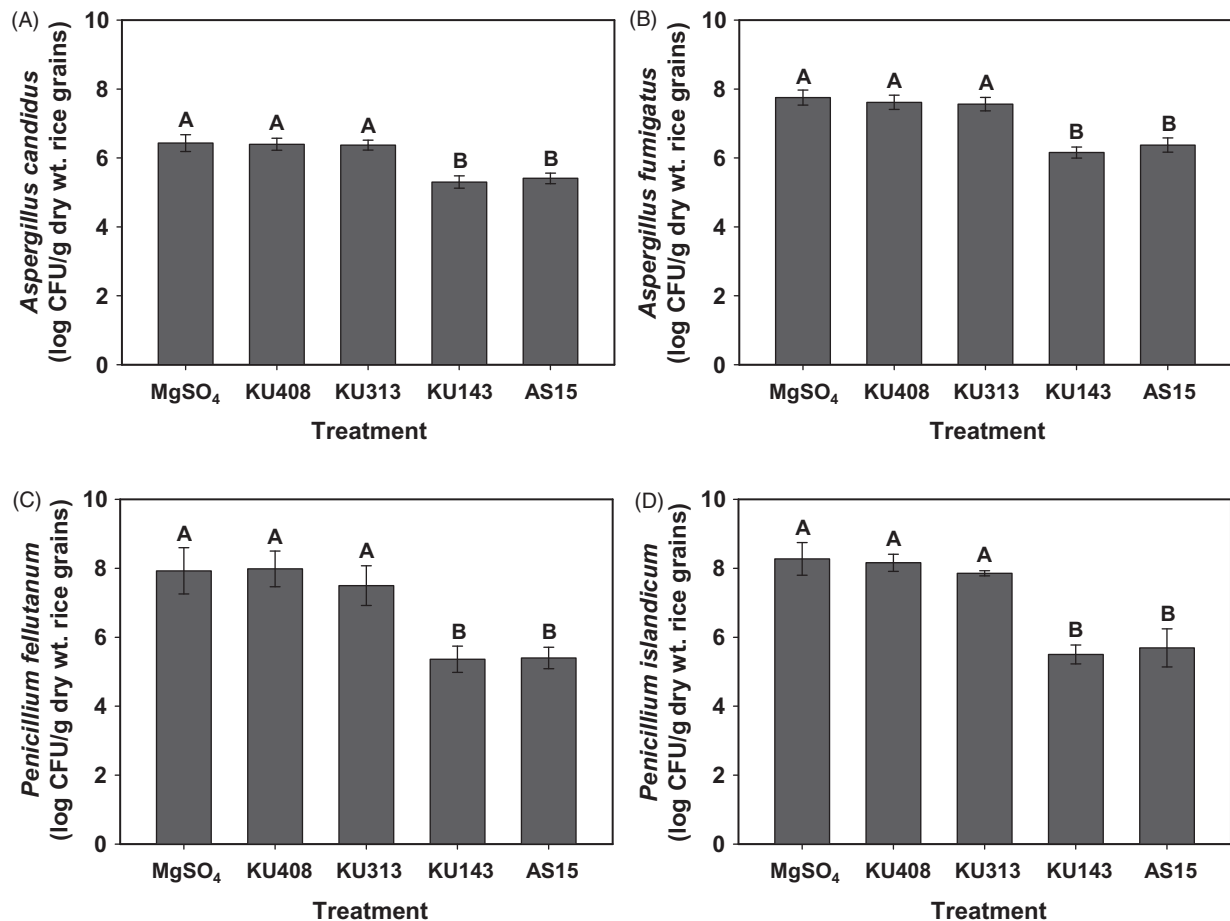
### 3.5. Volatile compounds produced by *B. megaterium* KU143 and *P. protegens* AS15

GC-MS analysis of volatiles produced by *B. megaterium* KU143 and *P. protegens* AS15 revealed 12 and 17 distinct peaks, respectively (Figure 4). The 12 peaks from *B. megaterium* KU143 identified using the best match in the Chemstation library were:

heptane [peak number (PN) 1], 2,5-dimethyltetrahydrofuran (PN 2), methylcyclohexane (PN 3), 3-hexanone (PN 4), 2-hexanone (PN 5), trimethyl [4-(1,1,3,3-tetramethylbutyl) phenoxy] silane (PN 6), 3-hexene-2,5-diol (PN 7), 2-bromohexane (PN 8), 2,4,4-trimethyl-1-pentanol (PN 9), 2,6-dimethylpiperazine (PN 10), 2,2-dimethyl-3-hexanol (PN 11), and 5-methyl-2-phenyl-1H-indole (PN 12). Similarly, the 17 peaks from *P. protegens* AS15 were identified as: heptane (PN 1), dimethyl disulfide (PN 2), 2,2,3-trimethylpentane (PN 3), 4-methylheptane (PN 4), 3-hexanone (PN 5), 2-hexanone (PN 6), 2,4-dimethyl-1-heptene (PN 7), 3-methylcyclopentanol (PN 8), 3,3-dimethyloctane (PN 9), 2,6-dimethylnonane (PN 10), 1-(2-methylbutyl)-1-(1-methylpropyl)cyclopropane (PN 11), 2-butyl-1-octanol (PN 12), undecane (PN 13), 1,3-di-*tert*-butylbenzene (PN 14), 4-trifluoroacetoxyhexadecane (PN 15), 2-isopropyl-5-methyl-1-heptanol (PN 16), and isotridecanol (PN 17). However, no distinct volatile compounds were detected in the TSB control (Figure 4).

## 4. Discussion

Biological control of plant pathogens or deleterious storage fungi with microbial antagonists or metabolites is an environmentally sound alternative to conventional chemical control, especially at the postharvest stage, when the use of agricultural chemicals is potentially undesirable for animal or human health and development of fungicide resistance [39]. The investigation of biocontrol or antimicrobial characteristics of antagonistic microbes is often important for understanding biocontrol mechanisms to get better control efficacy of the antagonists and for isolating biological compounds that are effective against deleterious grain fungi [40]. In our previous studies [20,34], we selected three antagonistic bacterial strains, *B. megaterium* KU143,



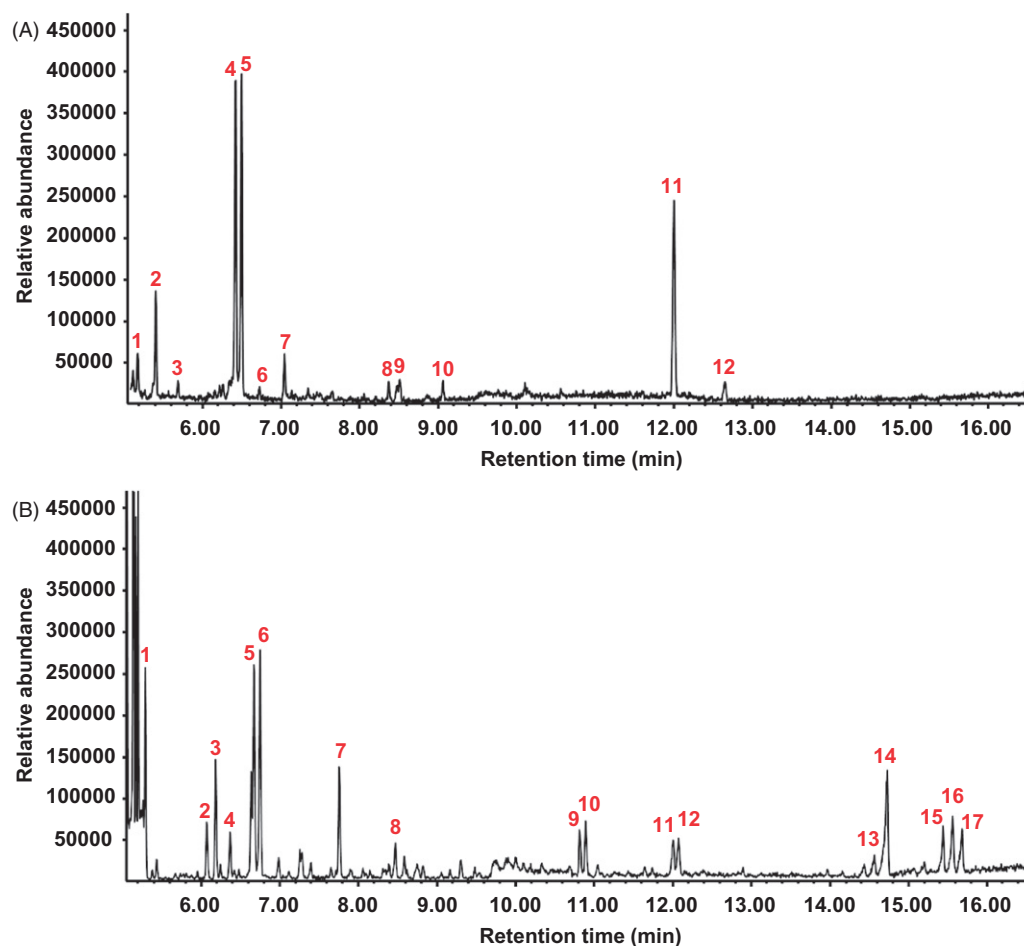
**Figure 3.** Antifungal activities of the volatiles produced by antagonistic bacterial strains, *Microbacterium testaceum* KU313, *Bacillus megaterium* KU143, and *Pseudomonas protegens* AS15, and negative control strain *Sphingomonas aquatilis* KU408 against populations of (A) *Aspergillus candidus* AC317, (B) *Aspergillus fumigatus* AF8, (C) *Penicillium fellutanum* KU53, and (D) *Penicillium islandicum* KU101, 7 d after treatment in unhulled rice grains on the I-plates. Bacterial strains or 10 mM MgSO<sub>4</sub> solution (untreated control) were smeared on one side (nutrient agar) of the I-plates and the other side (rice grains) was inoculated with a conidial suspension of the tested fungi, 24 h after bacterial treatment. Fungal populations in rice grains were assessed on DG18 after 4 d of incubation at 28 °C. Different capital letters on the vertical bars indicate significant ( $p < .05$ ) differences between treatments according to the least significant difference test. CFU: colony-forming unit.

*M. testaceum* KU313, and *P. protegens* AS15, from a total of 460 strains isolated from rice grains, against *A. flavus*. The selected strains exhibited significant *in vitro* and *in vivo* antifungal activity against *A. flavus*. These strains were efficient colonizers of the surface of rice grains and good competitors of *A. flavus* of the grains, as shown in our previous study [20]. Furthermore, the strains exhibited an inhibitory effect against aflatoxin production in rice grains. Among these strains, *P. protegens* AS15 could degrade aflatoxin B<sub>1</sub> and utilize the toxin for bacterial growth in nutrient-deficient conditions. In this study, the selected antagonistic strains were examined if they could show a broad spectrum of antifungal or biocontrol activity against *A. candidus*, *A. fumigatus*, *P. fellutanum*, and *P. islandicum*, which are deleterious grain fungi predominant in stored rice. Consequently, we found that these strains inhibited the growth and development of all the tested fungi on media and rice grains.

The investigation of biocontrol activity of the strains in this study revealed that they could

produce extracellular secondary metabolites with antifungal activity against the tested fungi from stored rice grains. These strains inhibited mycelial growth of the fungi in dual-culture assays. The cell-free culture filtrates also suppressed conidial germination, germ tube elongation, and mycelial growth of the fungi. Previously, viable cells and supernatants of *Lactobacillus* spp. have been shown to inhibit the growth of *A. flavus* and to reduce germination rates and germ-tube elongation of fungal conidia [41]. Our results from culture filtrates of the antagonistic strains in this study were consistent with previous observations by Manna et al. [20], which showed that the culture filtrates significantly inhibited the growth of *A. flavus* in rice grains. Reddy et al. [42] also showed that the culture filtrates of several biocontrol agents were inhibitory against *A. flavus* and aflatoxin production in rice grains, while filtrates from *Pseudomonas fluorescens* and *Rhodococcus erythropolis* produced the most suppressive activities against *A. flavus* growth and aflatoxin production. Similarly, in this study, culture filtrates from





**Figure 4.** Gas chromatographs of the volatiles produced by (A) *Bacillus megaterium* KU143 and (B) *Pseudomonas protegens* AS15. Antagonistic bacterial strains were cultured in tryptic soy broth (TSB) at 28 °C for 24 h. Volatile compounds from *B. megaterium* KU143 were identified as: heptane [peak number (PN) 1], 2,5-dimethyltetrahydrofuran (PN 2), methylcyclohexane (PN 3), 3-hexanone (PN 4), 2-hexanone (PN 5), trimethyl [4-(1,1,3,3,-tetramethylbutyl) phenoxy] silane (PN 6), 3-hexene-2,5-diol (PN 7), 2-bromohexane (PN 8), 2,4,4-trimethyl-1-pentanol (PN 9), 2,6-dimethylpiperazine (PN 10), 2,2-dimethyl-3-hexanol (PN 11), and 5-methyl-2-phenyl-1H-indole (PN 12). Volatile compounds from *P. protegens* AS15 were identified as: heptane (PN 1), dimethyl disulfide (PN 2), 2,2,3-trimethylpentane (PN 3), 4-methylheptane (PN 4), 3-hexanone (PN 5), 2-hexanone (PN 6), 2,4-dimethyl-1-heptene (PN 7), 3-methylcyclopentanol (PN 8), 3,3-dimethyloctane (PN 9), 2,6-dimethylnonane (PN 10), 1-(2-methylbutyl)-1-(1-methylpropyl)-cyclopropane (PN 11), 2-butyl-1-octanol (PN 12), undecane (PN 13), 1,3-di-*tert*-butylbenzene (PN 14), 4-trifluoroacetoxyhexadecane (PN 15), 2-isopropyl-5-methyl-1-heptanol (PN 16), and isotridecanol (PN 17). However, no distinct volatile compounds were detected in the TSB control.

*P. protegens* AS15 exhibited the most effective antifungal activity of the tested bacterial strains against the growth of all the tested fungi.

One of the potential biocontrol methods is bio-rational approach, which uses antifungal compounds produced by antagonistic microbes, particularly when the direct application of the biocontrol agents is not feasible due to practical limitations [43]. For example, biocontrol species *Bacillus subtilis* has been shown to produce antifungal peptides (peptidolipid iturins), such as bacillomycin and mycosubtilin. These compounds can inhibit not only spore germination of mycotoxigenic *Aspergillus*, *Fusarium*, and *Penicillium*, but also the fungal contamination of stored grains, such as corn, peanuts, and cottonseeds [43–45]. Several other compounds from *Bacillus pumilus* are also known to have inhibitory activities against *Mucoraceae* and *Aspergillus*, including *A. flavus* and *A. fumigatus* [46,47]. Dikin et al. [48] reported that

the supernatant of a *M. testaceum* strain contained aminopyrrole, phenazine, phenylpyrrole, and pyrrol-nitrin, which could inhibit the growth of *Schizophyllum commune*. Meanwhile, Zhang et al. [49] observed that cell-free culture filtrates from a strain of *B. subtilis* reduced the growth and spore germination of *A. flavus*, and possessed bacillomycin-like active compounds, which showed complete inhibition of *A. flavus* in peanuts [49]. Thus, further investigation is needed for purification and characterization of the antifungal compounds produced by the bacterial strains tested in this study.

Volatile compounds from antagonistic bacteria are one of the important traits for their biocontrol or antifungal activity [50]. For example, Fernando et al. [51] attempted to identify bacterial volatiles from *Pseudomonas aurantiaca*, *Pseudomonas chlororaphis*, *Pseudomonas corrugata*, and *Pseudomonas fluorescens*. They presented several compounds, such as

benzothiazole, cyclohexanol, *n*-decanal, dimethyl trisulfide, 2-ethyl-1-hexanol, and nonanal, as having inhibitory activities against ascospore germination and mycelial growth of *Sclerotinia sclerotiorum*. Other studies [30,31] demonstrated that 2,4-di-*tert*-butylphenol produced by *Flavobacterium johnsoniae* inhibited mycelial growth, sporulation, and spore germination of *Colletotrichum acutatum* and *Phytophthora capsici*. Volatiles from *Bacillus amyloliquefaciens* are also known to have antifungal activity, inhibiting the growth and spore germination of *Fusarium oxysporum* f. sp. *cubense* [52]. In our previous study [21], volatiles from *B. megaterium* KU143 and *P. protegens* AS15 inhibited mycelial growth, sporulation, conidial germination, and aflatoxin production by *A. flavus* on media and rice grains. The biocontrol activity of the volatiles from these two strains was also observed in this study against the growth and development of *A. candidus*, *A. fumigatus*, *P. fellutanum*, and *P. islandicum*. However, *M. testaceum* failed to produce significant antifungal volatiles against the tested fungi, except for *A. fumigatus* in medium tests.

In the GC-MS analysis of bacterial volatiles, 12 and 17 volatile compounds produced by *B. megaterium* KU143 and *P. protegens* AS15, respectively, were identified. Among these compounds, 5-methyl-2-phenyl-1H-indole from *B. megaterium* KU143 and 2-butyl 1-octanal, dimethyl disulfide, 2-isopropyl-5-methyl-1-heptanol, and 4-trifluoroacetoxyhexadecane from *P. protegens* AS15 were reported as having antifungal or antimicrobial activities [53–59]. Derivatives and related compounds of 5-methyl-2-phenyl-1H-indole were previously found to possess biological activity against not only bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* but also fungi such as *Aspergillus niger* and *Aspergillus oryzae* [54,57]. Wang et al. [59] identified volatiles from biocontrol species *Streptomyces albidoflavus*, in which inhibited *Aspergillus* spp. and *Fusarium moniliforme*. Among the volatiles, dimethyl disulfide completely inhibited the mycelial growth and sporulation of *F. moniliforme*. Similarly, Li et al. [53] observed that volatiles from *Streptomyces globisporus* had antifungal activity against *Penicillium italicum* on citrus fruits. When the identified compounds were tested for antifungal activity, it was found that dimethyl disulfide inhibited mycelial growth, sporulation, and conidial germination of *P. italicum*. Additionally, 2-butyl 1-octanal was identified from the biocontrol agent *Trichoderma harzianum* [58]. Antimicrobial compound 4-trifluoroacetoxyhexadecane was identified from secondary metabolites of the leaves and bark of *Naringi crenulate* [55]. Previously, 2-isopropyl-5-methyl-1-heptanol was reported as one of the prevailing compounds from the leaves of *Feronia*

*elephantum*, an Indian tree that has been used for medical purposes [56].

Taken together, the present results show that the tested bacterial strains, *B. megaterium* KU143, *M. testaceum* KU313 and *P. protegens* AS15, possess a broad spectrum of antifungal activities against *Aspergillus* and *Penicillium* spp., which are predominant in stored rice grains. These strains produced extracellular secondary metabolites with inhibitory activity against mycelial growth, conidial germination, and germ-tube elongation of the tested fungi. In addition, *B. megaterium* KU143 and *P. protegens* AS15 could produce volatiles with antifungal activity against the tested fungi. Among the identified volatile compounds, the antifungal compound, 5-methyl-2-phenyl-1H-indole, was produced by strain KU143, whereas the antimicrobial compounds, 2-butyl 1-octanal, dimethyl disulfide, 2-isopropyl-5-methyl-1-heptanol, and 4-trifluoroacetoxyhexadecane, were produced by strain AS15. Therefore, the bacterial strains in this study, especially *B. megaterium* KU143 and *P. protegens* AS15, may be potential biocontrol agents against *Aspergillus* and *Penicillium* species during rice grain storage.


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## Disclosure statement

No potential conflict of interest was reported by the authors.

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