



# An antifungal compound secreted by termite workers, phenylacetic acid, inhibits the growth of both termite egg-mimicking fungus and entomopathogenic fungi

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## Abstract

Social insects live in closely related family groups but face risks of intrusion and infection by pathogenic and parasitic microbes. To cope with the microbes invading their nests and feeding sites, social insects produce various types of antimicrobial substances. Subterranean termites occupy microbe-rich decaying wood and soil at high density, expanding their nest area by exploring and feeding on wood outward from the royal chamber (room for kings and queens). Although antimicrobial agents have been identified in many termite species, few studies have investigated those used by foraging workers in decaying wood under development, which is richer in microbes than the well-sterilized royal chamber and its surroundings. Here, we report that phenylacetic acid, an antifungal aromatic compound, is secreted by foraging workers of the Japanese subterranean termite *Reticulitermes speratus*. The compound was detected by gas chromatography–mass spectrometry analysis of ethyl acetate extracts of shelter papers infested with the workers, and antimicrobial tests demonstrated that it inhibits the germination and/or mycelial growth of the entomopathogenic fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and the termite egg-mimicking fungus *Athelia termitophila*. Our study provides new insights into the antimicrobial defense mechanisms of termites, including by combining different types of antimicrobial substances secreted by different castes, and thus the survival strategy of entomopathogenic and parasitic fungi in termite nests.

**Keywords** *Reticulitermes* · Termites · Antifungal substance · Entomopathogens · GC–MS

## Introduction

The remarkable diversity and ecological success of social insects have been attributed to their ability to cope with infectious microbes invading their nests and feeding sites (Traniello et al. 2002; Cremer et al. 2018). Because social insects live in dense clusters with closely related family

members susceptible to infections by the same pathogen (Cremer et al. 2007), infectious diseases can easily spread between group members, in contrast to solitary insects (Meunier 2015). Because larger social groups tend to have higher disease burdens, social insects have evolved prophylaxis and control measures to maintain hygiene in response to the potentially intense selection pressures posed by parasites and pathogens (Anderson and May 1979; Fefferman et al. 2007; Cremer and Sixt 2008).

Termites are social insects that form colonies of hundreds of thousands of close relatives, maintaining dense nests in microbe-rich wood and soil (Tsunoda et al. 1999). Maintaining a protected environment in their nests, by preventing pathogen invasion and growth, is thus a critical task of termites (Cremer et al. 2007). While physical removal of pathogens such as allogrooming is a basic hygiene behavior (Eggleton 2011), termites have developed the ability to produce and secrete a variety of antimicrobial substances. In the evolutionary transition in termites from one-piece nesting life type (the nest itself provides food for termites) to

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multiple-site nesting life type (nests of a single colony are interconnected by belowground tunnels and aboveground shelter tubes) (Abe 1987; Shellman-Reeve 1997), termite workers have experienced increased selective pressure for immune substances such as gram-negative-binding proteins (GNBPs), as they forage pathogen-rich environments outside of the nest. Such selective pressure could have enhanced social mechanisms of defense that afforded effective front-line external protection (Korb et al. 2015; Bulmer and Stefano 2022). Besides allogrooming and secretion of antimicrobial proteins, various antimicrobial organic compounds are used as protective substances: for example,  $\alpha$ -pinene and limonene from *Nasutitermes* species (Zhao et al. 2004), trinervitane and *n*-hexanoic acid from *Zootermopsis* species (Rosengaus et al. 2004), and naphthalene, an antiseptic and an anthelmintic (Middleditch et al. 1981) found in the inner wall of the nest of *Coptotermes formosanus* Shiraki (Chen et al. 1998). Antifungal and antibacterial proteins (lysozymes, termicin, spingerin, and Gram-negative-binding proteins) have also been identified in many termite species (Bulmer and Crozier 2006; Terrapon et al. 2014; Mitaka et al. 2017a). However, as none of these substances is effective against all pathogenic microorganisms, termites must have multiple antimicrobial substances with different antimicrobial spectra to combat the various types of pathogenic microorganisms in the environment.

*Reticulitermes* termites including *Reticulitermes speratus* are classified as multiple-site nesting termites (Yanagihara et al. 2018). In a colony of *R. speratus*, a royal chamber, i.e., a room of kings and queens, is located deep in one of the nest trees (Yanagihara et al. 2018) and this chamber is kept at low risk of pathogen infection due to the antifungal protection provided by the antifungal volatile queen pheromone (mixture of *n*-butyl-*n*-butyrate and 2-mehtyl-1-butanol) (Matsuura and Matsunaga 2015) and antibacterial egg recognition pheromone (lysozyme) (Matsuura et al. 2007). On the other hand, the foraging area, i.e., the area away from the royal chamber and where soldiers protect nest entrance (Yanagihara et al. 2018) and workers often forage for new wood food (Crosland et al. 1997), are considered to be microbe-rich environment compared with the sanitized royal chamber. Therefore, workers and soldiers in the foraging area are at higher risk of disease transmission than those around the royal chamber. Previous studies revealed that soldier pheromone ( $\beta$ -elemene) secreted from soldiers suppress the growth of entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* (Mitaka et al. 2017b), and mellein contained in bodies of workers and soldiers in the foraging area also suppress the growth of these fungi (Mitaka et al. 2019). The antifungal activity of these compounds is not so strong and only retards mycelial growth of entomopathogenic fungi. Nevertheless, foraging areas where workers come and go are usually clean and few areas of microbial growth are found (Cremer et al. 2007). This suggests

that some antimicrobial substance may be present not only in the body of individual termite but also in the inside of the nest.

In addition to pathogenic microorganisms, termite-egg-mimicking fungus 'termite ball' are also parasitic in egg piles in the nests of *Reticulitermes* termites (Yashiro and Matsuura 2007). Termite balls are the sclerotia of *Athelia termitophila* (Maekawa et al. 2020). They mimic termite eggs both morphologically (Matsuura 2005) and chemically (Matsuura et al. 2009) and thereby be protected from desiccation and other microorganisms by egg-caring behavior of workers. Once the care of workers becomes absent, termite balls start to germinate (Matsuura et al. 2000; Komagata et al. 2022) and can consume surrounding eggs (Matsuura and Matsunaga 2015). Since the interaction is parasitic, in that it is beneficial for the fungus but costly for the host termites, the germination and growth of termite balls are usually inhibited throughout the nests in the filed colonies (Matsuura 2006). The volatile queen pheromone, which is emitted by queens and eggs (Matsuura et al. 2010), is known to be able to inhibit germination and mycelial growth of termite balls (Matsuura and Matsunaga 2015). However, the queen pheromone should be absent or in very low concentration in foraging area far from the royal chamber, and  $\beta$ -elemene and mellein emitted by workers and soldiers have no inhibitory effect against termite balls (Mitaka et al. 2017b, 2019). Therefore, it is expected that compounds other than the queen pheromone inhibit growth of entomopathogenic microorganisms and termite balls in the interior surface of foraging areas.

In this study, ethyl acetate extracts of secretions on shelter papers infested with *R. speratus* workers were analyzed by gas chromatography–mass spectrometry (GC–MS), which led to the identification of phenylacetic acid. Tests of its activity against the growth of the egg-parasitic fungus *A. termitophila* and the entomopathogenic fungi *M. anisopliae* and *B. bassiana* were conducted. The antibacterial activities of this compound against several species of entomopathogenic (*Bacillus subtilis*, *Bacillus thuringiensis*, and *Serratia marcescens*) and opportunistic (*Micrococcus luteus*, and *Pseudomonas aeruginosa*) bacteria were examined as well, based on previous laboratory bioassays demonstrating the pathogenicity of these entomopathogen to termites (Shimizu and Yamaji 2002; Singha et al. 2010; Omoya and Kelly 2014).

## Materials and methods

### Termite collection

Five *R. speratus* colonies were collected from secondary forests in Japan as follows: colony TI003 was collected from Ryuo-cho, Shiga Prefecture, in April 2018; colony

190124C was collected from Amarube-cho, Kameoka, Kyoto Prefecture, in January 2019; colony 190409B was collected from Shimoyada-cho, Kameoka, Kyoto Prefecture, in April 2019; and colonies 200622A and 200622B were collected from Ohmiya-Shakadani, Kita-ku, Kyoto, Kyoto Prefecture, in June 2020.

## Ethics

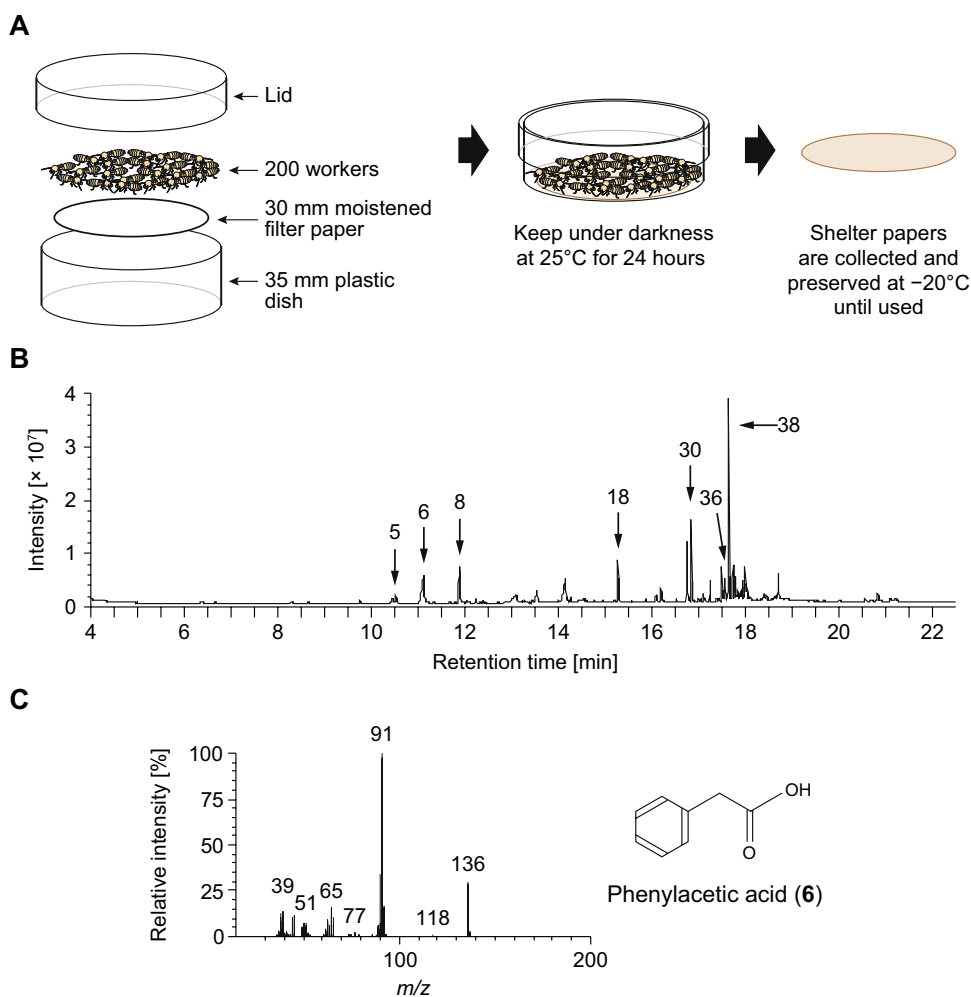
No specific permits were required for the described field activities. Specific permission was also not required to access or sample the termite colonies, as they were collected from unprotected public lands. This study did not involve endangered or protected species. However, in Japan, phenylacetic acid is classified as a raw material for stimulant production; we obtained the necessary designation certificate by the governor of Kyoto Prefecture to allow its purchase from FUJIFILM Wako Pure Chemical Corp., Osaka, Japan.

## Preparation of shelter papers

Substances secreted by termite workers into the inner walls of their nest were trapped using shelter papers (Fig. 1A). Two hundred workers from a colony were placed in a 35 mm dish lined with a filter paper (30 mm diameter, 0.26 mm thickness; Advantec No. 2; Toyo Roshi Kaisha, Ltd, Tokyo, Japan), with 20 dishes prepared from each of the five colonies (TI003, 190124C, 190409B, 200622A, and 200622B). The papers were moistened with 150  $\mu$ L distilled water and the dishes were incubated at 25 °C. Shelter papers were collected from each dish every 24 h for 7 days and preserved in a freezer at  $-20^{\circ}\text{C}$  for use in the analyses.

To extract the chemical compounds (including a variety of molecules ranging from non-polar to highly polar molecules) released by the termites, the shelter papers ( $n=200$ ) from each colony were placed in 200 mL glass jars and extracted with 200 mL ethyl acetate ( $\geq 99.5\%$  purity, FUJIFILM Wako Pure Chemical Corp.) for 24 h. As a control, unused filter papers ( $n=200$ ) were also extracted under the same condition. The resulting extracts were concentrated

**Fig. 1** Chemical profile of the shelter papers infested by workers of *R. speratus*. **A** Schematic drawing of the rearing dish used to prepare the shelter papers. **B** Representative gas chromatography result of the extract prepared from termite shelter papers (colony 200622B). Numbers associated with arrows indicate different compounds: 1-(2-butoxyethoxy)-ethanol (**5**), phenylacetic acid (**6**), 2-(2-butoxyethoxy)acetic acid (**8**), palmitic acid (**18**), *n*-tricosane (**30**), 2-methyltricosane (**36**), and *n*-pentacosane (**38**). Peaks without arrows mean colony-specific compounds. **C** Mass spectrum and structure of phenylacetic acid are shown



to 1 mL using a rotary evaporator and then subjected to GC–MS analyses for chemical identification.

### GC–MS analysis

GC–MS analyses were performed on a JMS-Q1500GC mass spectrometer (JEOL Ltd., Tokyo, Japan) combined with an Agilent Technologies 7890B GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-1MS column (30 m × 250 μm × 0.25 μm, Agilent Technologies). The column temperature was held at 50 °C for 5 min, then increased from 50 to 300 °C at 20 °C/min, and held for 5 min. Then, one μL sample was manually injected using an injector in splitless mode, helium as the carrier gas (1 mL/min), and an injection port temperature maintained at 250 °C. MS data were obtained under the following conditions: 50 μA ionization current, 70 eV ionization energy, 2 kV accelerating voltage, a 40–500 *m/z* scan range, and 2,300 amu/s scan rate. Both GC and MS systems were controlled using an msPrimo system controller ver. 1.06 (JEOL Ltd.). The data were analyzed using the software Escore ver. 2.04 (JEOL Ltd., <https://www.jeol.co.jp/en/products/detail/JMS-Q1500GC.html>). Candidate compounds were predicted based on the Kovats index information from the NIST Chemistry WebBook, Standard Reference Database Number 69 (<https://doi.org/https://doi.org/10.18434/T4D303>) and the mass spectrum similarities from the EI MS library of the NIST/EPA/NIH Mass Spectral Library 2011 (NIST11). Phenylacetic acid was identified according to its Kovats index and mass spectrum, based on a comparison with the commercial standard. Kovats indices of candidate compounds was calculated using the retention times of phenylacetic acid standard (98% purity, FUJIFILM Wako Pure Chemical Corp.) and a mixture of C9–C33 *n*-alkanes' standards. The concentration of phenylacetic acid in each extract was calculated by comparison with an external standard curve (regression  $R^2 = 0.94$ ). Since the samples used for GC–MS analysis were adjusted for concentration to be 0.2 filter paper equivalents/μL, the amount of phenylacetic acid per filter paper was calculated based on the calculated concentration of it in each extract. Finally, we calculated the phenylacetic acid secreted by one worker over a 24-h period, taking into account that the phenylacetic acid contained in the filter paper was secreted by 200 workers over a 24-h period.

### Antifungal tests

The inhibitory effect of phenylacetic acid on mycelial growth of *A. termitophila*, *M. anisopliae*, and *B. bassiana* was tested. The strain of *A. termitophila* used in this bioassay was isolated from the nest of *R. speratus* as described in a previous study (Mitaka et al. 2019). *M. anisopliae* (NBRC31961)

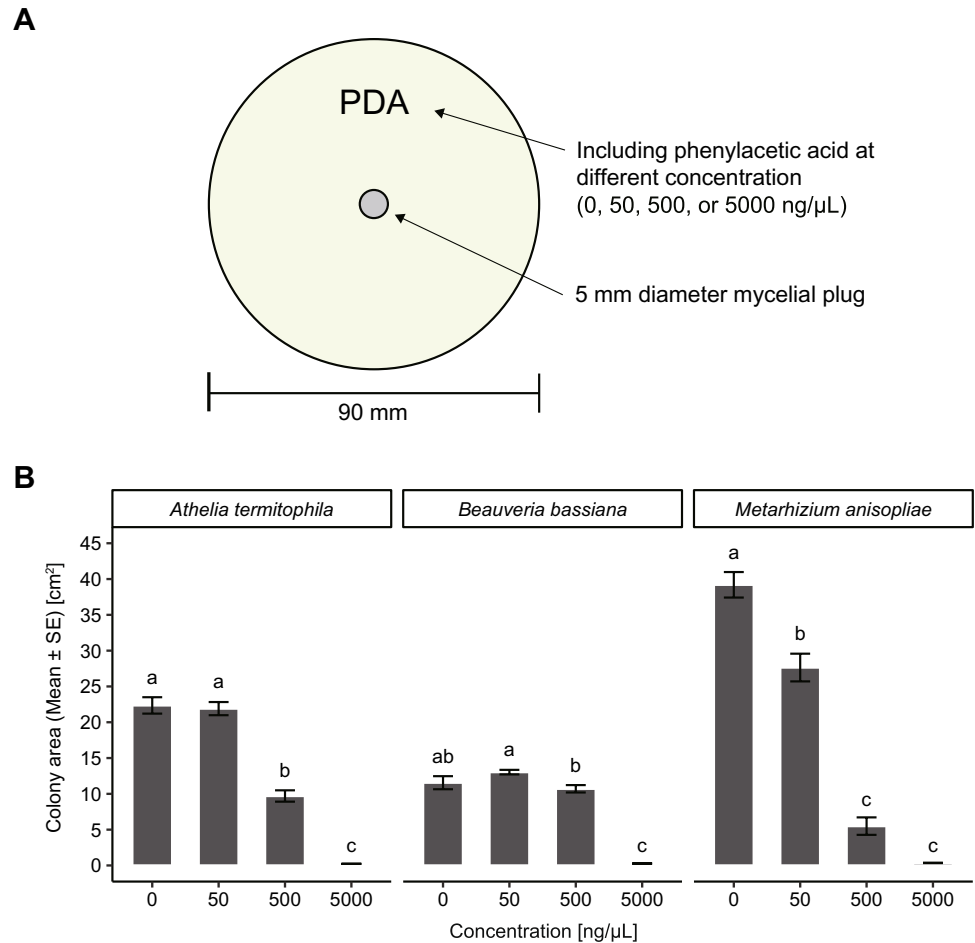
and *B. bassiana* (NBRC103721) strains were provided by the Biological Resource Center (NBRC, National Institute of Technology and Evaluation, Tokyo, Japan) and cultured on potato-dextrose agar (PDA) plates at 28 °C. The assay was performed by placing a 5 mm diameter plug of growing mycelia from each fungal culture in the center a Petri dish (90 × 15 mm) containing phenylacetic acid at concentrations of 0, 50, 500, and 5,000 ng/μL (Fig. 2A). The Petri dishes were wrapped with two layers of Parafilm and incubated at 28 °C for 14 days. Five replicates were prepared for each treatment of each fungus. The size of the mycelia was measured by taking vertical photographs of each dish every 7 days after inoculation using a digital camera (tg-6, Olympus, Japan). The colony area in cm<sup>2</sup> was determined by counting the total number of pixels in the fungal colony area using ImageJ software (US National Institutes of Health, Bethesda, MD, USA) (Schneider et al. 2012).

The inhibitory effect of phenylacetic acid on the spore germination rate of entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) was also tested by modifying a previously conducted method (Hywel-Jones and Gillespie 1990). We did not assess *A. termitophila*, because this species does not produce amorphous spores under culture conditions (Maekawa et al. 2020). Spores of fungi plates were washed by pipetting 10 mL of 0.05% Tween 80 onto the plate and scraping off the spore with a spatula. The spore suspension was transferred to a 15-mL Eppendorf tube, shaken, and filtered through sterilized gauze to remove aggregates. The spore concentration was determined with a haemocytometer (Thoma chamber; Erma, Saitama, Japan) and diluted to a final concentration of 1–2 × 10<sup>7</sup> spores/mL. The 100 μL spore suspension was spread on to each of five replicate PDA agar plates (90 × 15 mm) including phenylacetic acid at concentrations of 0, 50, 500, or 5,000 ng/μL. After seeding, the plates were incubated at 28 °C for 24 h, and 300 spores on the plates were randomly observed with the inverted microscope at ×200 magnification (DM IL LED, Leica, Tokyo, Japan). Spores whose germ tubes were more than half the length of the spore diameter were classified as germinated. Germination rates were calculated as follows: the number of germinated spores / the number of total spores observed (= 300).

### Antibacterial tests

The antibacterial activity of phenylacetic acid against *B. subtilis*, *B. thuringiensis*, *M. luteus*, *P. aeruginosa*, and *S. marcescens* was tested as described previously (Zhu et al. 2011). Strains of *B. subtilis* (NBRC3009), *B. thuringiensis* (NBRC13865), *M. luteus* (NBRC16250), and *P. aeruginosa* (NBRC3080) were provided by the NBRC. The *S. marcescens* strain used in the bioassay was isolated from the nest of *R. speratus* following a procedure developed

**Fig. 2** Inhibitory effect of phenylacetic acid against the mycelial growth of fungi. **A** Experimental set-up. Phenylacetic acid was added to the PDA medium (90 mm diameter, 5 mm height) to achieve a final concentration of 0, 50, 500, and 5,000 ng/ $\mu$ L of phenylacetic acid. **B** The results of antifungal tests. Values denote the mean  $\pm$  standard error of mean of five replications. Different letters indicate significant differences (ANOVA followed by Tukey's HSD test,  $P < 0.05$ ). Phenylacetic acid inhibited *A. termitophila*, *B. bassiana*, and *M. anisopliae* at a concentration  $\geq 500$  ng/ $\mu$ L, 5,000 ng/ $\mu$ L, and 50 ng/ $\mu$ L, respectively



in a previous study (Inagaki and Matsuura 2018) and cultured on lysogeny broth (LB, Nakarai Tesque, Kyoto, Japan) agar plates at 30 °C.

The agar diffusion assay was performed by spreading 100  $\mu$ L bacterial inoculum ( $1-2 \times 10^8$  CFUs/mL) on an LB agar plate. Then, 40  $\mu$ L aqueous phenylacetic acid at concentrations of 0, 50, 500, and 5,000 ng/ $\mu$ L (equivalent to 0, 2,000, 20,000, and 200,000 ng of phenylacetic acid) was added to a 7 mm (diameter) well punched in the center of the plate. As the negative control, distilled water was added to the well. Ten replicates were prepared for each treatment of each bacterium. The plates were wrapped with two layers of Parafilm and incubated at 30 °C for 24 h. After incubation, the major and minor axes of the inhibition zone were measured using a digital caliper, and the means of two measurements were used to calculate the apparent area of the inhibition zone. Because this area included the area of the well, the practical area of the inhibition zone was calculated by subtracting the area of the well from the apparent area.

### Statistical analysis

All statistical analyses were performed using R software v. 4.2.2 (R Core Team 2016). Fungal colony area (the size of mycelia) was compared using an analysis of variance (ANOVA) followed by Tukey's HSD test ( $P < 0.05$ ). Spore germination rate was compared using a generalized linear mixed model (GLMM). The germination rate was regarded as the response variable, assuming a binomial distribution. Concentrations were used as an explanatory variable. Replications were used as a random effect. Likelihood ratio tests (LRTs) were conducted to test the significance of each explanatory variable, and Tukey's HSD tests were conducted to test for differences between levels of a significant fixed effect. In the antibacterial tests, the area of the inhibition zone was compared using an ANOVA followed by Tukey's HSD test ( $P < 0.05$ ).

## Results

### Chemical profile of termite shelter papers

We detected a total of 60 compounds (**1**–**60**) from the extracts of filter papers (including both shelter papers and unused filter papers) by GC–MS. Among these, only seven compounds were consistently detected in the extracts of the shelter papers derived from the five *R. speratus* colonies (Fig. 1B and Table 1): 1-(2-butoxyethoxy)-ethanol (**5**), phenylacetic acid (**6**), 2-(2-butoxyethoxy) acetic acid (**8**), palmitic acid (**18**), *n*-tricosane (**30**), 2-methyltetracosane (**36**), and *n*-pentacosane (**38**). 2-(2-butoxyethoxy) acetic acid was also detected in the extract of unused filter papers (Table 1). Therefore, a total of six compounds (**5**, **6**, **18**, **30**, **36**, and **38**) was considered to be derived from termites. 1-(2-butoxyethoxy)-ethanol (**5**) is commonly used as solvents or preservatives in siloxane (used as stationary phase in GC columns) production (Scherer et al. 2019), suggesting that this compound was contaminant. Palmitic acid (**18**) is used for an aggregation pheromone in *R. speratus* (Mitaka et al. 2020), and *n*-tricosane (**30**), 2-methyltetracosane (**36**), and *n*-pentacosane (**38**) are cuticular hydrocarbon components in *R. speratus* (Takematsu and Yamaoka 1999; Mitaka et al. 2020). Therefore, it is unlikely that these five compounds function as antimicrobial compound. However, only phenylacetic acid is reported to be an antimicrobial compound able to inhibit the growth of fungi and bacteria (Kim et al. 2008; Zhu et al. 2011; Fernández-Marín et al. 2015). We, therefore, focused on this compound. The amount of phenylacetic acid extracted per shelter paper was  $1991.569 \pm 1231.247$  ng (mean  $\pm$  SEM). Because 200 workers were used in this procedure, we estimated that the amount of phenylacetic acid secreted in 24 h was 9.958 ng/worker.

### Estimation of the phenylacetic acid concentration in the foraging area

Field colonies of *R. speratus* contain a median and maximum of 24,500 and 451,800 workers, respectively (Takata et al. 2023). In the subterranean termite *Coptotermes formosanus*, 20% of the workers are estimated to be foragers, and the foraging worker rate is maintained regardless of changes in total colony size and demographic composition (Lee et al. 2022). Assuming the same proportion for *R. speratus*, the median and maximum numbers of foraging workers in field colonies of *R. speratus* are 4,900 and 90,360, respectively. If all foraging workers pass through an area of the same size as the shelter paper (706.858 mm<sup>2</sup>

[ $\approx 15 \text{ mm} \times 15 \text{ mm} \times \pi$ ]) where they secrete phenylacetic acid for 24 h, the median and maximum concentrations of phenylacetic acid soaked into the spot would be 48,794.200 ng (= 9.958 ng per worker  $\times$  4,900 workers) and 899,804.900 ng (= 9.958 ng per worker  $\times$  90,360 workers), respectively.

### Antifungal activity of phenylacetic acid

To test the inhibitory effects of phenylacetic acid on fungal mycelial growth, colonies of entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) and the egg-mimicking termite ball fungus (*A. termitophila*) were exposed to authentic phenylacetic acid at different concentrations (Fig. 2A). Inhibitory effects against *M. anisopliae* were observed at 50–5,000 ng/ $\mu$ L (ANOVA,  $df=3$ ,  $F_{3,16}=160.2$ ,  $P<0.001$ , Tukey's HSD test,  $P<0.05$ ; Fig. 2B), against *A. termitophila* at 500–5,000 ng/ $\mu$ L (ANOVA,  $df=3$ ,  $F_{3,16}=162.6$ ,  $P<0.001$ , Tukey's HSD test,  $P<0.05$ ; Fig. 2B), and against *B. bassiana* at only 5,000 ng/ $\mu$ L (ANOVA,  $df=3$ ,  $F_{3,16}=113.2$ ,  $P<0.001$ , Tukey's HSD test,  $P<0.05$ ; Fig. 2B).

The inhibitory effect of phenylacetic acid on the spore germination of entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) was also tested by exposing the spores to the compound at different concentrations. The inhibitory effect was observed at 50–5,000 ng/ $\mu$ L in both *M. anisopliae* (LRT,  $df=3$ ,  $\chi^2=7179.3$ ,  $P<0.001$ , Tukey's HSD test,  $P<0.05$ ; Fig. 3) and *B. bassiana* (LRT,  $df=3$ ,  $\chi^2=4970.1$ ,  $P<0.001$ , Tukey's HSD test,  $P<0.05$ ; Fig. 3).

### Antibacterial activity

To determine whether phenylacetic acid inhibits bacterial growth, colonies of entomopathogenic bacteria (*B. subtilis*, *B. thuringiensis* and *S. marcescens*), a typical Gram-positive bacterium (*M. luteus*), and a typical Gram-negative bacterium (*P. aeruginosa*) were exposed to authentic phenylacetic acid at different concentrations. The compound inhibited the growth of all bacteria only at the highest tested concentration of 5,000 ng/ $\mu$ L (ANOVA,  $df=3$ ,  $F_{3,36}=84.30$  [*B. subtilis*], 128.60 [*B. thuringiensis*], 110.50 [*M. luteus*], 204.30 [*P. aeruginosa*], or 192.00 [*S. marcescens*],  $P<0.001$ , Tukey's HSD test,  $P<0.05$ ; Fig. S1).

## Discussion

In *R. speratus* colonies, foraging areas are a more microbe-rich environment than the royal chamber, such that foraging workers were predicted to secrete substances that inhibit microbial growth. Our GC–MS analysis revealed that foraging workers of *R. speratus* secrete phenylacetic acid (Fig. 1), initially identified as a plant-growth-promoting substance in

**Table 1** Compound list

No	Compound name	Kovats index	Colony TI003	Colony 190124C	Colony 190409B	Colony 200622A	Colony 200622B	Control paper
1	3-Hydroxy-butanoic acid	938	-	-	-	+	-	-
2	(Acetyloxy)-acetic acid	1012	-	-	-	+	-	-
3	2-Ethyl-hexanoic acid	1102	+	-	-	-	-	-
4	Benzoic acid	1180	-	-	-	-	+	-
<b>5</b>	<b>1-(2-Butoxyethoxy)-ethanol</b>	<b>1159</b>	+	+	+	+	+	-
<b>6</b>	<b>Phenylacetic acid</b>	<b>1221</b>	+	+	+	+	+	-
7	Phthalic acid	1259	+	-	+	-	-	-
<b>8</b>	<b>2-(2-Butoxyethoxy)acetic acid</b>	<b>1335</b>	+	+	+	+	+	+
9	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	1396	-	+	+	+	+	+
10	(E)-(2-ethyl-4-methyl-1,3-pentadienyl)-Benzene	1411	-	-	-	+	-	-
11	<i>n</i> -Tetradecanoic acid	1733	+	-	-	+	+	-
12	(1-Hexylheptyl)-benzene	1814	+	-	-	-	-	-
13	(1-Butylnonyl)-benzene	1824	+	-	-	-	-	-
14	(1-Propyldecyl)-benzene	1834	+	-	-	-	-	-
15	Mono-2-(2-methoxyethoxy)ethyl phthalate	1850	+	-	-	-	-	-
16	1-Hexadecanol	1867	-	+	-	-	-	+
17	Hexadecenoic acid*	1914	+	-	-	-	-	-
<b>18</b>	<b><i>n</i>-Hexadecanoic acid</b>	<b>1935</b>	+	+	+	+	+	-
19	9-Hexadecen-1-ol	2057	-	-	-	-	-	+
20	1-Hexadecanol	2080	-	+	-	-	-	-
21	Tributyl aconitate	2106	-	-	-	-	-	+
22	Vaccenic acid*	2117	+	-	-	+	+	-
23	1-Propene-1,2,3-tricarboxylic acid, tributyl ester	2128	-	+	-	-	-	-
24	<i>n</i> -Octadecanoic acid	2139	+	-	-	+	+	-
25	1-Docosanol	2168	-	-	-	-	+	-
26	Tributyl acetylcitrate	2214	+	+	+	-	-	+
27	Tricosene*	2278	+	-	-	-	+	-
28	Tricosene*	2282	-	-	+	-	-	-
29	Tricosene*	2286	-	+	-	+	-	-
<b>30</b>	<b><i>n</i>-Tricosane</b>	<b>2300</b>	+	+	+	+	+	-
31	11-Methyltricosane	2337	+	-	-	-	-	-
32	2-Methyltricosane	2366	+	-	-	+	+	-
33	(Z)-9-Octadecenamide	2345	-	+	-	-	-	+
34	<i>n</i> -Tetracosane	2400	+	-	+	+	+	-
35	3-Ethyl-5-(2-ethylbutyl)-octadecane	2412	-	+	-	-	-	-
<b>36</b>	<b>2-Methyltetracosane</b>	<b>2466</b>	+	+	+	+	+	-
37	1-Docosanol	2483	-	-	-	+	+	-
<b>38</b>	<b><i>n</i>-Pentacosane</b>	<b>2500</b>	+	+	+	+	+	-
39	Bis(2-ethylhexyl) phthalate	2510	+	-	-	+	+	-
40	11-Methylpentacosane	2517	+	+	-	+	+	-
41	3-Ethyltetracosane	2532	+	-	-	+	+	-
42	3-Methylpentacosane	2536	+	-	-	+	-	-
43	1,2-Cyclohexanedicarboxylic acid, cyclohexyl-methyl nonyl ester	2560	-	-	-	-	-	+
44	1,2-Cyclohexanedicarboxylic acid, cyclohexyl-methyl nonyl ester	2573	-	+	-	-	+	+
45	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2585	-	+	-	-	-	+
46	<i>n</i> -Hexacosane	2600	+	-	-	-	-	-
47	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2671	-	-	-	-	-	+

**Table 1** (continued)

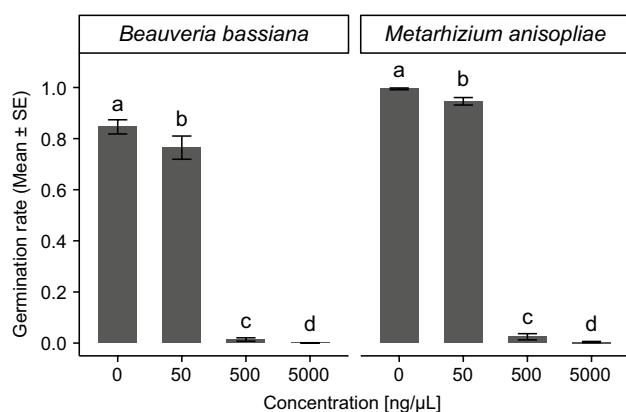
No	Compound name	Kovats index	Colony TI003	Colony 190124C	Colony 190409B	Colony 200622A	Colony 200622B	Control paper
48	1',1'-Dicarboethoxy-1 $\beta$ ,2 $\beta$ -dihydro-3'H-cycloprop[1,2]cholesta-1,4,6-trien-3-one	2703	–	–	+	–	+	–
49	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2711	–	–	–	–	–	+
50	Docosenamide*	2719	+	–	+	+	+	–
51	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2726	–	–	–	–	–	+
52	3-Ethyl-5-(2-ethylbutyl)-octadecane	2733	–	–	+	–	–	–
53	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2747	–	–	–	–	–	+
54	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2752	–	–	–	–	–	+
55	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2759	–	–	–	–	–	+
56	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	2771	–	–	–	–	–	+
57	<i>n</i> -Octacosane	2800	+	–	–	–	–	–
58	(3 $\beta$ ,5 $\beta$ )-Cholestan-3-ol	3116	–	–	–	+	+	–
59	(5 $\beta$ )-Cholestan-3-one	3134	+	–	–	–	–	–
60	Cholesterol	3158	–	–	–	+	+	–

+: Detected, –: not detected

**Bold:** compounds shared among the extracts of shelter papers from five colonies (but not included in the extract of control paper)

Control paper: the filter papers without termite workers

\*Position and geometry of the double bond not unambiguously determined



**Fig. 3** Inhibitory effect of phenylacetic acid against the spore germination of entomopathogenic fungi *B. bassiana* and *M. anisopliae*. Phenylacetic acid was added to the PDA medium (90 mm diameter, 5 mm height) to achieve a final concentration of 0, 50, 500, and 5,000 ng/μL of phenylacetic acid. Values denote the mean ± standard error of mean of five replications. Different letters indicate significant differences (GLMM followed by Tukey's HSD test,  $P < 0.05$ ). Phenylacetic acid inhibited both *B. bassiana* and *M. anisopliae* at a concentration  $\geq 50$  ng/μL

the 1930s but later shown to possess substantial antimicrobial activity in bacteria, fungi, algae, land plants, and insects (Fernández-Marín et al. 2015; Cook 2019). However, its antimicrobial effects against the entomopathogenic microorganisms of termites had not been investigated. The amount of phenylacetic acid secreted by *R. speratus* (9.958 ng per worker per 24 h according to the quantification done after

GC–MS measurement) is sufficient to suppress the mycelial growth of *M. anisopliae* and *A. termitophila* in their colonies. Assuming that all foraging workers in the field continually secrete it over a foraging area equivalent to the area of one shelter paper (706.858 mm<sup>2</sup>), then after 24 h, the cumulative median and maximum amounts of phenylacetic acid will be 48,794 ng and 899,805 ng, respectively. In PDA media with phenylacetic acid in our antifungal tests (Fig. 2A), the amount of this compound in an area equivalent to one shelter paper (assuming a thickness of 1 mm, that is, a volume of 706.858 mm<sup>3</sup> = 706.858 μL) is 35,343 ng for the 50 ng/μL treatment, 353,429 ng for the 500 ng/μL treatment, and 3,534,290 ng for the 5,000 ng/μL treatment, respectively. The results of mycelial growth tests demonstrated that phenylacetic acid inhibited mycelial growth of *M. anisopliae* at the 50–5,000 ng/μL treatments, *A. termitophila* at the 500–5,000 ng/μL treatments, and *B. bassiana* at the 5,000 ng/μL treatment in antifungal tests (Fig. 2B). This means that field termites would suppress the mycelial growth of *M. anisopliae* if the number of foraging workers were above the median, and the growth of both *M. anisopliae* and *A. termitophila* if the number of foraging workers reaches the maximum. The mycelial growth of *B. bassiana* would not be suppressed even at the maximum number of foraging workers. However, phenylacetic acid inhibited not only the spore germination of *M. anisopliae* but also that of *B. bassiana* at  $\geq 50$  ng/μL, especially at  $\geq 500$  ng/μL (Fig. 3), suggesting that termites would suppress the spore germination of *M. anisopliae* and *B. bassiana* if the number of foraging



workers reached the medium, and strongly suppress if the foraging workers reached the maximum.

Since the foraging worker rate is usually kept constant in termite colonies (Lee et al. 2022), it is unlikely that the number of foraging workers in a large colony changes throughout the year unless there is an accident that causes a drastic decrease in colony size, such as the loss of many workers at once. Therefore, it is considered that the foraging areas in a large colony always accumulate sufficient phenylacetic acid to suppress the spore germination and mycelial growth of *M. anisopliae* and *B. bassiana* and suppress the mycelial growth of *A. termitophila*. On the other hand, in the antibacterial tests, phenylacetic acid resulted in inhibition zones for all tested strains at the 5,000 ng/ $\mu$ L treatment, although the size of the zone was very small ( $\leq 180$  mm<sup>2</sup>, Fig. S1). Even with the maximum number of foraging workers secreting the compound in the field, bacterial growth would barely be suppressed. These results suggest that phenylacetic acid functions as an effective antimicrobial substance only in the developing range of the foraging area with high worker traffic, not in the undeveloped area with few workers.

*R. speratus* releases a variety of antimicrobial substances with different effects on microbial growth. Mellein, secreted by foraging workers and soldiers, inhibits the mycelial growth of *B. bassiana* but not that of *A. termitophila* (Mitaka et al. 2019). Several termite pheromones also have antimicrobial effects. For example, lysozyme, a component of termite egg recognition pheromone, has broad antibacterial activity against Gram-positive bacteria including *Bacillus* species (Matsuura et al. 2007), while queen pheromone, a mixture of n-butyl-n-butylate and 2-methyl-1-butanol, inhibits the germination and mycelial growth of *M. anisopliae*, *B. bassiana*, *Isaria farinose*, *Sclerotium tuliparum*, *Athelia rolfsii*, and *A. termitophila* (Matsuura and Matsunaga 2015). However, queen pheromone is secreted only by queens and eggs (Matsuura 2012). The internal structure of *Reticulitermes* termite nests is multi-layered (Yanagihara et al. 2018) and the radius of the foraging area can range from one meter to tens or even hundreds of meters (Vargo and Husseneder 2009). Therefore, the effective range of the antifungal activity of queen pheromone would be limited to the area around the royal chamber and its side egg chamber. In this work, phenylacetic acid secreted from foraging workers was shown to inhibit the mycelial growth of *M. anisopliae* and *A. termitophila*, with suppression involving the entire termite nest where many colony members are present and active. Our results suggest that each caste of *R. speratus* makes use of multiple antimicrobial substances in combination to inhibit the growth of pathogenic microorganisms.

Antimicrobial activity of phenylacetic acid was also reported in *Atta* leaf-cutting ants, and these ants secrete this compound from the metapleural gland to inhibit the spore germination and growth of pathogenic fungi

(Fernández-Marín et al. 2015). In that study, *B. bassiana* and *Metarhizium brunneum* were isolated from the ant nests but only the mycelial growth of *B. bassiana* was inhibited by phenylacetic acid. This contrasts with our bioassays, in which the growth of *M. anisopliae* but not *B. bassiana* was suppressed (Fig. 2). Thus, susceptibility to phenylacetic acid likely varies among fungal species within the same genus and among strains of the same species. Parallel to the competition between pathogens and termites, parasites and their host termites seem to be engaged in a coevolutionary arms race, in which parasites eventually gain resistance to antimicrobial substances. In open field environments, the lower relatedness among parasites within infected hosts leads to higher levels of within-host competition, which selects for higher parasite virulence (Frank 1994). The coexistence of multiple strains of parasitic fungi within the nests of social insects gives parasitic fungi a distinct advantage in the coevolutionary arms race between hosts and parasites (Yashiro et al. 2011). In response, termites are likely to use multiple antimicrobial substances, alone, or in combination, against parasitic microorganisms that may be costly to the colony. The process of acquiring resistance to termite-secreted antimicrobial substances in entomopathogenic and parasitic fungi by species or strain should be investigated in the future.

Although the site of the biosynthesis of phenylacetic acid in the termite body is still unknown, it is probably in the gut tissues. A previous study reported that *Reticulitermes flavipes* fed high amounts of xylan, a hemicellulose contained in wood, increased the production of phenylacetic acid in the gut (Brasseur et al. 2016). In the phenylalanine biosynthesis pathway of many organisms, phenethylamine is produced and subsequently metabolized to phenylacetic acid (Ramos and Filloux 2007). Thus, amino acid metabolism by gut symbionts of *R. speratus* may include the production of phenylacetic acid, which is then excreted. Its presence in termite feces may help maintain nest hygiene. In *Zootermopsis nevadensis*, acetate produced by gut symbionts and excreted by the termite suppresses the growth of *S. marcescens* (Inagaki and Matsuura 2018). The mechanism of action of acetate and other weak acids is considered to involve cytoplasmic acidification of pathogenic microorganisms, in turn inhibiting enzyme activity and amino acid transport (Hillenga et al. 1995; Lambert and Stratford 1999; Weber et al. 2012). Phenylacetic acid is also a weak acid such that it presumably inhibits pathogen activity by a similar mechanism. Further studies are needed to determine the site of phenylacetic acid biosynthesis and the mechanism underlying its inhibition of fungal growth.

In summary, *R. speratus* secrete phenylacetic acid into their nest materials, where it inhibits not only the mycelial growth of *A. termitophila* and *M. anisopliae* but also the spore germination of *M. anisopliae* and *B. bassiana*.

Thus, this compound is one of several antimicrobial compounds, such as the antimicrobial components of pheromones (Matsuura et al. 2007; Mitaka et al. 2017b) and the other antimicrobial agent (Mitaka et al. 2019), released by termites to protect their colonies. The simultaneous use of multiple antimicrobial substances enables termites to cope with a wide variety of parasitic microorganisms. Our work contributes to a more in-depth understanding of the defense mechanisms of termites against pathogenic and parasitic microorganisms.

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**Author contributions** M.N., T.I., Y.M. and K.M. designed experiments. Y.M. and T.I. collected termites. Y.M. performed GC-MS analysis. M.N., Y.M. and T.I. performed antimicrobial tests. M.N. and Y.M. wrote the manuscript. All authors provided feedbacks and edits.

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**Data availability** The data used in this study are available in the electronic supplementary materials.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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