Role of bacteria in maintaining the monoculture of *Termitomyces* on the fungus combs of *Odontotermes obesus*

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Certificate of Examination

This is to certify that the dissertation titled "**Role of bacteria in maintaining the monoculture of *Termitomyces* on the fungus combs of *Odontotermes obesus***" submitted by Mr. Abin Antony (Reg. No. MS14175) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rhitoban Ray Choudhury (Supervisor) Dr. N. G. Prasad Dr. Indranil Banerjee

Dated: April 26, 2019.

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rhitoban Ray Choudhury at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a confide record of original work done by me and all sources listed within have been detailed in the bibliography.

Abin Antony

April 26, 2019

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rhitoban Ray Choudhury

(Supervisor)

April 26, 2019

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Abin Antony

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ABSTRACT

A lineage of higher termites are known for fungus farming inside their mounds. They live in an obligate mutualism with fungus of genus *Termitomyces* from 30 Mya. Termites depend on their cultivar fungus for the digestion of lignocellulose and *Termitomyces* depend on termites for growth and protection. *Termitomyces* is cultivated as monoculture on a structure called fungus comb which is made up of partially digested plant materials passed through the gut of termites and asexual spores of *Termitomyces*. Termites then feed on to the symbiotic fungus buds which grow by degrading components and using those nutrients from the comb. *Pseudoxylaria* has been identified as one of the main antagonists of *Termitomyces.* They are prevalent in termite mounds and appear to be competing with *Termitomyces* for resources present in fungus combs. *Pseudoxylaria* species are inconspicuous in healthy mounds, but are observed to be present almost always in the mound and rapidly overgrow *Termitomyces* in the absence of termites. The process by which termites maintain and protect *Termitomyces* monoculture by selectively suppressing the growth of antagonistic fungi is still not understood.

Previous studies in fungus-growing termites have shown the presence of symbiotic bacteria which can produce antifungal compounds to selectively inhibit the growth of antagonistic fungi. But, it is not proved that the termites are using these symbionts in this process of selective inhibition. This study is constituted of three parts: a culture-dependent microbiome study to isolate and identify different bacteria present in *Odontotermes obesus* colony, a behavioral study to observe how termites respond to externally introduced *Pseudoxylaria* and antifungal activity assays to check for antifungal activity exhibited by bacteria obtained from different experiments.

Bacteria which belong to 15 genera and 5 classes were isolated and identified from different termite samples of *Odontotermes obesus* colony. In the behavioral study, termites were observed to cover externally introduced *Pseudoxylaria* with soil in the presence of fresh fungus comb. Bacteria obtained from experiments were found to have no inhibitory effects on the growth of *Pseudoxylaria* in antifungal activity assays. But, these experiment provide an example of biologically relevant situations in which potential defensive symbiotic bacteria are presumably abundant and relatively easy to find.

INTRODUCTION

Termites

Termites are insects which belong to the order Blattodea (Inward *et al.,* 2010) and infraorder Isoptera. They are hemimetabolous insects, having life stages as egg, nymph and adult. About 3,106 species of living and fossil termites have been recognized and classified (Krishna *et al.,* 2013). They are categorized into two groups- higher and lower termites, based on their evolution, anatomy and behavior. Termites are detritivores and they utilize cellulose and lignocellulose from decaying plant material by different means. Lower termites which include families Hodotermitidae, Mastotermitidae, Rhinotermitidae, Serritermitidae, Kalotermitidae and Termopsidae harbor symbiotic protozoans in their hindgut which helps in the digestion of lignocellulose (Krishna & Weesner, 1970). Instead, higher termites which belong to the subfamily Macrotermitinae use the help of a symbiotic fungus for the utilization of lignocellulose.

Eusociality

Eusociality is the highest or extreme level of social organization in animals. Characteristics like cooperative brood care, overlapping generations within a colony and reproductive altruism defines eusociality in animals (Honeycutt, 1992; Crespi & Yanega, 1995; Gadagkar, 1993; Wilson, 1971, 2005). Social insect colonies are considered as super organisms based on the extent to which individuals appear to efficiently operate as a unit for maintenance, perpetuation and reproduction of the colony (Queller & Strassmann, 1998). Eusociality is mostly observed and studied in Hymenopterans and in termites. Eusocial behavior is also observed in other organisms like *Synalpheus* snapping shrimp, halicted bee *Lasioglossum duplex*, adult erotylid beetles of the genus *Pelaphacus*.Ambrosia beetle *Austroplatypus incompertus etc.*, (Duffy *et al*., 2007; Sakagami & Hayashida, 1960; Costa, 2006; Kent & Simpson, 1992; Nowak *et al*., 2010).

Eusociality and caste system in termites

Termites are eusocial organisms. Evolution of eusocial termites can be traced back to upper Jurassic or lower cretaceous period. Evidences suggest that termites are descended from ancestors which used wood for food and shelter (Thorne *et al*., 2000).) Division of labor is prominent in termite colonies. All castes are physiologically distinct from one another. Castes include workers, soldiers, nymphs and alates, where male and female alates have reproductive capacity while workers and soldiers are sterile. Reproducing individuals are concentrated in reproduction only, workers in foraging and brood care and soldiers in defense. It is proposed that termite ancestors likely had a life history based on colony formation in which foraging occur only within the host wood (Thorne *et al*., 2000). Challenging habitats and competition for food led to attacking of neighboring colonies. This situation exerted pressure on the need of a defense system for the colony which led to the evolution of division of labor. High morphological distinction between the castes, especially of soldiers indicates that inter-colonial battles influenced the evolution of modern sterile termite soldier weaponry and behaviors (Thorne *et al.,* 2003). It is estimated that in Isoptera, true workers evolved 3 times which had developmental and reproductive options (Abe, 1990; Higashi *et al.,* 1991; Myles, 1988; Myles and Nutting, 1988; Noirot and Pasteels, 1987, 1988).

Fungus farming in insects

There are 3 orders of insects in which fungus farming is reported and described. Ants, termites and ambrosia beetles have independently evolved agriculture in their course of evolution (Muller *et al*., 2005). Evidences suggest that agriculture has evolved seven times in ambrosia beetles (Farrel *et al*., 2009), but only once in termites and ants (Aanen *et al*., 2002; Mueller *et al*., 2001).

Fungus farming in these insects are similar in many aspects. Obligate dependency on crops for food, providing optimal growth conditions for crops, cultivation on modified substrates, strict and continuous monitoring, sustainable harvesting of crop, protection of crop from diseases and antagonists *etc.*, are some of the common features of agriculture among these organisms (Muller *et al*., 2005).

All fungus farming ant systems are observed to have the presence of atleast four symbionts for its maintenance: the fungus-growing ants; their fungal cultivars (Mueller et al. 1998, Munkacsi et al. 2004); mutualistic antibiotic-producing Actinomycete

bacteria (Currie et al. 1999); parasitic fungi of genus *Escovopsis* (Currie *et al.*, 1999; Currie *et al.*, 2003). These ants prepare fungus gardens by spreading a mixture of their cultivar fungus and beneficial microbes onto processed substrate. They are observed to exhibit active weeding behavior to remove contaminant microbes and fungus garden diseases. Mutualistic interactions between organisms involved is thought to be a main factor which provides stability to this system.

Ambrosia beetles construct tunnel systems in wood in order to cultivate and maintain their fungal crop (Wood, 1982). The term ambrosia refers to the cultivar fungus which are grown on the walls of galleries constituted by the tunnel system. Fungus gardens of ambrosia beetles which belong to a monophyletic tribe Xyleborini are not pure monocultures. It is composed of an assemblage of mycelial fungi, bacteria and yeast (Batra, 1966; Haanstadt & Norris 1985). These beetles are observed to be able to control the growth of cultivar fungi and the composition of its multiple fungal species to an extent (Beaver, 1989; French & Roeper, 1972; Kingsolver & Norris, 1977; Roeper *et al*. 1980) in ways which are not fully understood.

Fungus farming in termites

About 330 species of higher termites which belong to the subfamily Macrotermitinae are reported for the presence of agriculture (Batra and Batra, 1966). Evidences suggest that origin of fungus farming termites happened in African rain forests and they are found throughout old world tropics (Aanen & Eggleton, 2005).

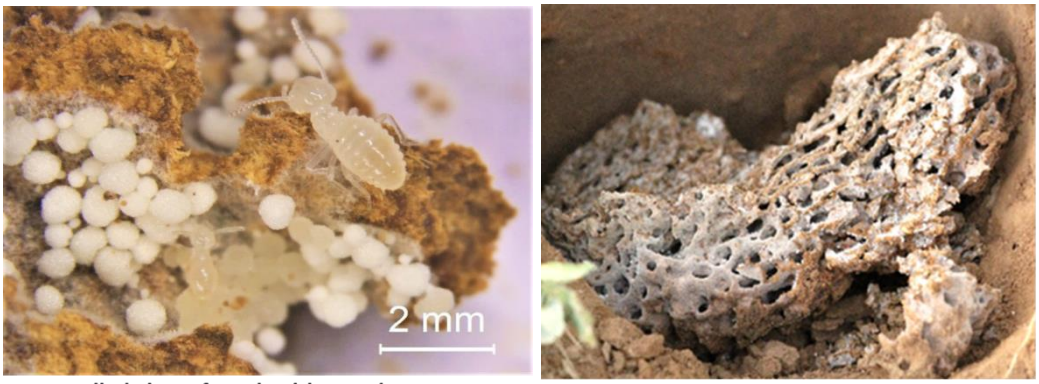

Fig 0.1: Fungus comb of *Odontotermes obesus*

Picture courtesy: Evogen lab

Fungus farming termite colonies are founded by a pair of alates. After winged-alates emerge from the mounds, they participate in a nuptial flight inorder to find a mate. They lose their wings after this process and initiate the founding of a new mound on a suitable substratum. The female alate starts to lay eggs after they seal themselves in a chamber made of soil (this will later become what is known as the royal chamber). Mature workers of the first brood start to forage, build the mound and fungus farming is established inside the mound. The process of transmission of the cultivar fungus into a newly founded mound is not clearly understood.

There is no diurnal fluctuation observed in temperature and relative humidity in mounds of termites which belong to the genus *Odontotermes.* Variation of these throughout the year was found to be in a narrow range (4 °C and 4%) (Agarwal, 1980). Macrotermes mounds are found to be so complex and well designed so that it can retain heat during cold and dissipate heat during summer (Rajagopal, 1982). Well engineered ventilation system helps to maintain temperature and humidity conditions inside the mound with less variation throughout the year, which is essential for the survival of *Termitomyces.*

Termitomyces is cultivated on a structure called fungus comb which is made up of partially digested plant materials passed through the gut of termites and asexual spores of *Termitomyces* (Lefe`vre *et al.,* 2000). Termites then feed on to the symbiotic fungus buds which grow by degrading components and using those nutrients from the comb (Rohrmann, 1978; Hyodo *et al.,* 2000; Hyodo *et al.,* 2003).

Termite- *Termitomyces* mutualism

Mutualistic association between termites and *Termitomyces* can be traced back to 30 Mya (Aanen & Eggleton, 2005). This mutualism is obligate for both the partners. Termites depend on their cultivar fungus for food and *Termitomyces* depend on termites for growth and protection. Evolution of fungus farming happened only once in termites. Establishment of this mutualistic symbiosis is a result of long-term coevolution between both the partners and this association remains unbroken. Neither the fungus growing termites, nor their symbiont *Termitomyces* species have abandoned this association to go back to their free-living state since then (Aanen *et al.,* 2002).

Lignocellulose is one of the main component in wood and plant matter. It consists of Cellulose (28–50%), hemicellulose (20–30%) and lignin (18–30%), with the cellulose and hemicellulose polymers tightly bound to lignin (Breznak and Brune, 1994). Cellulose digestion becomes easy and enhanced when lignin is degraded. This lignin degradation has been suggested as the main role of *Termitomyces* in some termites (Hyodo et al. 2000). Being a nitrogen-rich food source is another widely suggested role of *Termitomyces* in lot of other species of termites (Rouland-Lefèvre *et al.*, 1991; Hyodo *et al.*, 2000).

Termites provide suitable conditions for the growth and protection of their cultivar *Termitomyces.* Temperature and high humidity conditions inside the mound is suitable for the growth of fungi. Successful fungus farming is expected to rely on effective defending of invading competitors and diseases too. There is a lot of organisms which are potential parasites and contaminants of fungus combs. Mites and nematodes are reported to be common parasites of the fungus gardens, which feed on the fungal nodules and contaminate the fungus combs with other fungal spores and microbes (Mueller and Gerardo, 2002). Soldier caste constituting the defense system of the colony, can prevent the entrance of intruders into the mound. Directed allo-grooming (directed at individuals exposed to parasites) is observed to be effective in removal of parasites from insect cuticle (Rosengaus et al. 1998; Yanagawa et al. 2008; Walker and Hughes, 2009). The low pH of the fungus comb helps to prevent the growth of bacteria, otherwise comb appears to be a favorable substrate for microbial activity (Thomas, 1987). Comb substrates passes through termite gut before its incorporation to the fungus comb. This can be a potential screening mechanism which differentially affects the survival of mutualistic and non-mutualistic fungi or other microbes, thereby taking care of a subset of antagonistic bacteria and fungi.

Antagonistic fungi

Being grown as a monoculture itself is predicted to make *Termitomyces* prone to invasion, competition and exploitation in the absence of termites. Some potential invading competitors of *Termitomyces* has been identified and described. Ascomycete fungus of genus *Xylaria* (Family- Xylariaceae) has been observed as a potential antagonist of *Termitomyces* (Sands, 1969). *Pseudoxylaria*, a sub-genus of *Xylaria* has been found to be a monophyletic clade which comprises all the *Xylaria* species

associated with fungus farming termites (Guedegbe *et al.*, 2009; Visser *et al.*, 2009; Hsieh *et al.*, 2010). They are prevalent in termite mounds and appear to be competing with *Termitomyces* for resources present in fungus combs. *Pseudoxylaria* species are inconspicuous in healthy mounds, but are observed to be present almost always in the mound and rapidly overgrow *Termitomyces* in the absence of termites.

Fig 0.2: Fungus combs infected by *Pseudoxylaria*

Picture courtesy: Evogen lab

Presence of fruiting bodies of *Pseudoxylaria* in abandoned termite mounds is also reported (Rogers, 2000; Rogers *et al.*, 2005). It has been proposed that *Pseudoxylaria* can be a stowaway which adopts a sit-and-wait strategy to survive in termite mounds (Visser *et al.*, 2011). Large overlapping in carbon source usage of *Termitomyces* and *Pseudoxylaria* has been reported. One-to-one interaction studies between *Termitomyces, Pseudoxylaria* and their free-living relatives showed significant difference between interaction of *Termitomyces* and *Pseudoxylaria* with each other than with each other's free-living relatives. Growth of both fungi was found to be less together than growing separately (Visser *et al.*, 2011). All these evidences confirm the competition between these fungi for resources.

Aspergillus, Penicillium, and Trichoderma are also reported as common microfungi which infect the fungus comb, compete with and affect the growth of the symbiotic fungus *Termitomyces* in Macrotermitinae termites (Wood & Thomas, 1989; Gullan & Cranston, 2010). These evidences strongly point towards the extent of competition faced by *Termitomyces* inside the termite mounds and the negative effect it may have on the productivity of fungus gardens. So, termites are expected to have evolved

strategies to suppress the growth and propagation of *Pseudoxylaria* and other antagonistic fungi.

Defense against antagonistic fungi

The presence of termite workers is observed to be playing a role in the defense strategies employed by termites against antagonistic fungi. It has been widely observed that the growth of *Pseudoxylaria* is initiated in mounds in the absence of termite workers, suggesting active inhibition of *Pseudoxylaria* by termite workers in mound (Shinzato *et al.,* 2005; Visser *et al.,* 2011). As the passage of substrate material through termite gut before its incorporation to fungus comb is obligate, this process itself can be a potential screening mechanism which allows the selective inhibition of antagonistic fungi (Nobre *et al.,* 2011). Chemical secretions from termites, with antifungal activity are also potential candidates involved in termite defense mechanisms (Lamberty *et al.,* 2001; Fuller, 2007). Workers are reported to have the ability to discriminate between *Termitomyces* and *Pseudoxylaria* using olfactory cues (Katariya *et al.,* 2017*).* This information provides scope for a behavioral defense mechanism which involves active weeding of antagonistic fungi. Defense mechanisms mediated by mutualistic symbionts also can be a potential way to resist and inhibit the growth of antagonistic fungi in fungus gardens.

Bacteria as defensive symbionts

Bacteria are found to have mutualistic association with fungus growing insects for defense purposes. Several species of Actinobacteria has been reported to occur as defensive symbionts in insects. In *Philanthus* species of European beewolves, symbiotic Actinobacteria harbored in adult's antennae helps in protecting the wasp larvae from fungal infections, mediated by compounds which suppress fungal growth (Kaltenpoth *et al.,* 2005; Kroiss *et al.,* 2010). *Streptomyces* bacteria has been reported to mediate the selective inhibition of an antagonistic fungus of the mutualistic fungus of *Dendroctonus frontalis* (Southern pine beetle) (Scott *et al.,* 2008). *Allomerus* species of fungus growing ants are found to be associated with Actinobacteria which produce antifungal compounds, for protection against antagonistic fungi (Seipke *et al.,* 2012).

Previous works in fungus growing termites *Odontotermes* and *Macrotermes* has suggested the presence of a potential mutualistic defensive symbiont *Bacillus.*

Secretion containing a compound called Bacillaene, produced by *Bacillus* species residing in termite gut and fungus comb, was found to selectively inhibit the growth of antagonistic fungi like *Trichoderma* and *Pseudoxylaria* but not *Termitomyces* (Mathew *et al.*, 2011; Um *et al* ., 2013) . But no studies in fungus growing termites have shown that the fungus-growing termites are actually using symbiotic bacteria for maintaining the monoculture of *Termitomyces* by inhibiting the growth and propagation of antagonistic fungi.

Objectives of this study

This study is based on a hypothesis that fungus-growing termites are using the help of symbiotic bacteria for maintaining the monoculture of *Termitomyces* in their colonies by inhibiting the growth and propagation of antagonistic fungi. Objectives of the study are the following:

- Identify different bacteria present in *Odontotermes obesus* colony using culture-dependent methods.
- Observe the response of termite workers towards externally introduced *Pseudoxylaria.*
- Find potential bacterial candidates among the cultivable microbiome of termite colony, which is possibly playing the role of a defensive symbiont and helps in maintaining the monoculture of *Termitomyces.*

CHAPTER 1

Culture-dependent microbiome study

This part of the study includes isolation and identification of different bacteria present in *Odontotermes obesus* colony in IISER Mohali campus, Punjab (Latitude: 30.7046486 m, Longitude: 76.7178726 m and elevation 316m (1037ft)).

1.1 Methods

Identification of termite and fungus

This study was conducted using samples majorly from one *Odontotermes obesus* mound named as H7. Termites and fungus comb were collected from the mound and *Pseudoxylaria* was obtained from fungus comb incubated without termites. Collected termites were washed twice with autoclaved water and were subjected to DNA isolation and identification. Fresh comb was incubated (temperature- 28 °C, Relative humidity- 85%) in the absence of termites for 2 days in order to obtain antagonistic fungus *Pseudoxylaria.* Genomic DNA isolation and identification was done using fungal mycelia obtained from infected comb.

DNA isolation

Termites

- Washed termite samples were taken in 1.5 ml Micro centrifuge tube (MCT). 200µL lysis buffer, 5µL Proteinase K (22mg/ml) and 5 µL SDS were added and sample was crushed using sterile pestle, followed by incubation at 60 ˚C in water bath for 2 hours.

Lysis buffer	100
5.0 M Tris HCl	2.0 ml
5 M NaCl	0.2 ml
0.5M EDTA	2.0 ml

Components of Lysis buffer

- 250 µl of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) was added and mixed, followed by centrifugation for 5 min at 4˚C at 12700 rpm.

- The topmost aqueous layer was collected and invert mixed with 250 µl of 24:1 Chloroform: Isoamyl alcohol, followed by centrifugation for 5 min at 4˚C at 12700 rpm.
- Aqueous layer was collected, chilled Isopropanol (70% volume of the sample) with 0.1 volume of Sodium acetate was added followed by incubation at room temperature for 45 minutes.
- After incubation, samples were centrifuged for 5 min at 4˚C at 12700 rpm and the obtained pellet was washed with ethanol followed by air drying.
- The resultant pellet was suspended and dissolved in 100 µl of 1X TE buffer.

Fungus

- Fungal genomic DNA isolation was carried out by Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method (Doyle & Doyle, 1987).
- Fungal mycelia was crushed in a MCT after adding 600µL modified CTAB buffer using sterile pestle.
- 4µL fresh RNAase (20 mg/ml) was added and incubated at 65°C for 30min followed by vigorous vortexing.

CTAB	2% W/V
NaCl	1.42 M
EDTA	20 mM
Tris	100 mM
β-Mercaptoethanol	1 µL

Components of CTAB buffer

- 600 µL of Phenol: chloroform: Isoamyl alcohol (25:24:1) was added and centrifugation at 12700 rpm for 15 min was carried out (this step was repeated for attaining better DNA quality).
- The topmost aqueous layer was collected and invert mixed with 600 µL of 24:1 Chloroform: Isoamyl alcohol, followed by centrifugation for 5 min at 4˚C at 12700 rpm.
- Aqueous layer was collected, chilled Isopropanol (70% volume of the sample) with 0.1 volume of Sodium acetate was added followed by incubation at room temperature for 45 minutes.
- After incubation, samples were centrifuged for 5 min at 4˚C at 12700 rpm and the obtained pellet was washed with ethanol followed by air drying.

- The resultant pellet was suspended and dissolved in 100 µL of 1X TE buffer.

Identification and phylogenetic analysis

The quality and quantity of obtained DNA was determined using Nanodrop spectrophotometer. Amplification of DNA was carried out using PCR followed by Agarose (1%) gel electrophoresis. For termite samples, PCR was performed using following primers (Ohkuma *et al.,* 2003) specific for mitochondrial DNA of *CO II* subunit.

- Forward primer - **COII-F1 (5'-GGDCAYCAATGRTRYTGAAG-3')**
- Reverse primer - **COII-B2 (5'-AGTACTTGCTTTCAGTCATC-3')**

For fungal samples, PCR was performed using universal fungal primers specific for ITS region - ITS4 and ITS5 (White *et al.,* 1990).

- Forward primer – **ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')**
- Reverse primer – **ITS4 (5'- TCCTCCGCTTATTGATATGC-3')**

Sequenced DNA was analyzed and identified using NCBI BLAST (Altschul *et al.,* 1990). Similar sequences from BLAST search and an outgroup sequence was used for phylogenetic analysis. Sequence alignment was done using CLUSTAL W (Thompson *et al.,* 1994) followed by Bayesian analysis (using Mr. Bayes) to construct phylogenetic tree.

Microbiome survey

Termite and comb samples were collected from *Odontotermes obesus* mound within IISER Mohali campus. Collected termites of different castes were washed twice with autoclaved water, crushed in 1X PBS and serial dilutions were streaked on LB agar and PDA plates. Same was done with fresh fungus comb except washing. Samples were used on the same day of collection. Bacterial colonies were isolated and sub-cultured based on morphological characteristics of the colony (color, texture, shape of border *etc*.). Pure cultures were used for DNA extraction by heating bacterial colonies dissolved in 1X TE at 95 °C for 3 minutes. DNA quantification was done using Nanodrop spectrophotometer and these samples was preserved at -20˚C. Bacterial samples were preserved as a 1:1 mixture of liquid culture of bacteria (in LB) and 50%

glycerol at -80 °C. Extracted DNA were used in PCR to amplify 16S rRNA region using forward primer 27F and reverse primer 1517R (Jiang *et al.*, 2006).

- Forward primer – **27F (5'- AGAGTTTGGATCMTGGCTCAG -3')**
- Reverse primer – **1517R (5'- ACGGCTACCTTGTTACGACIT -3')**

Amplified DNA products were sent for sequencing and results were analyzed using NCBI BLAST.

1.2 Results and Discussion

Identification of termite and fungus

Termite samples from H7 mound turned out to be ***Odontotermes obesus.*** Antagonistic fungus obtained from comb was identified as ***Pseudoxylaria*** of genus ***Xylaria*** of ***Xylariaceae*** family. Phylogram was prepared using similar sequences and an outgroup. *Microtermes obesi* and *Nemania* sequences were used as outgroups for termite and fungus sequences respectively. Phylograms are shown below

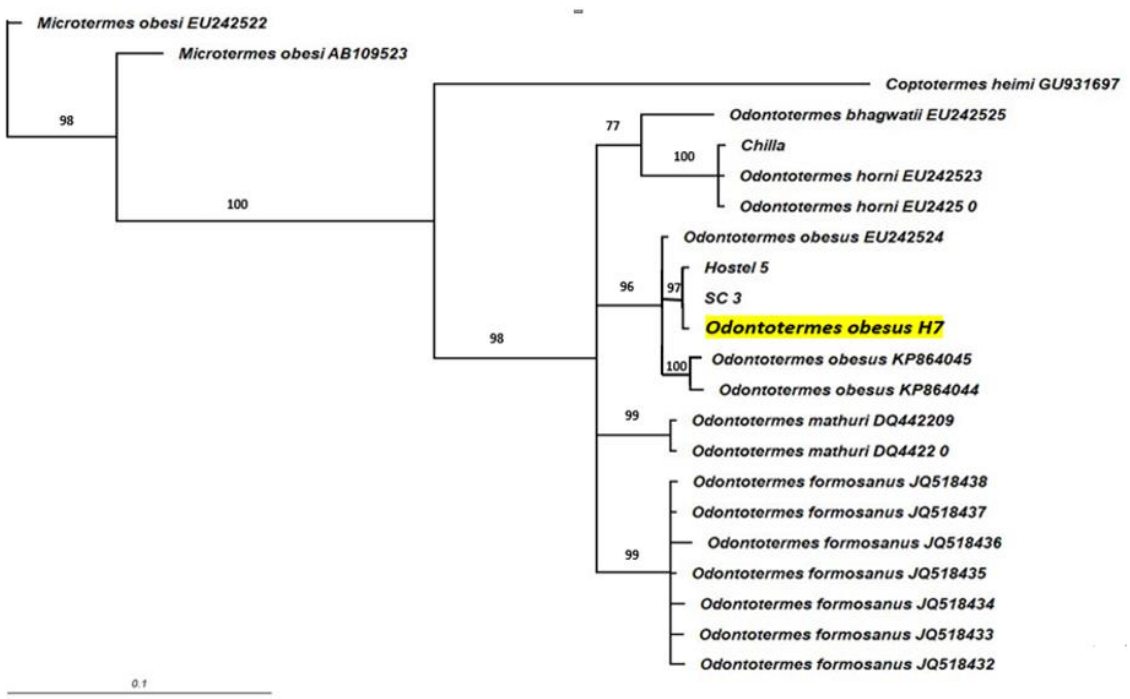

Fig 1.1 Phylogram prepared using identified termite sequence - (highlighted in yellow).

Note: Chilla, Hostel 5 and SC3 are previously identified *Odontototermes obesus* samples obtained from other mounds within IISER Mohali campus.

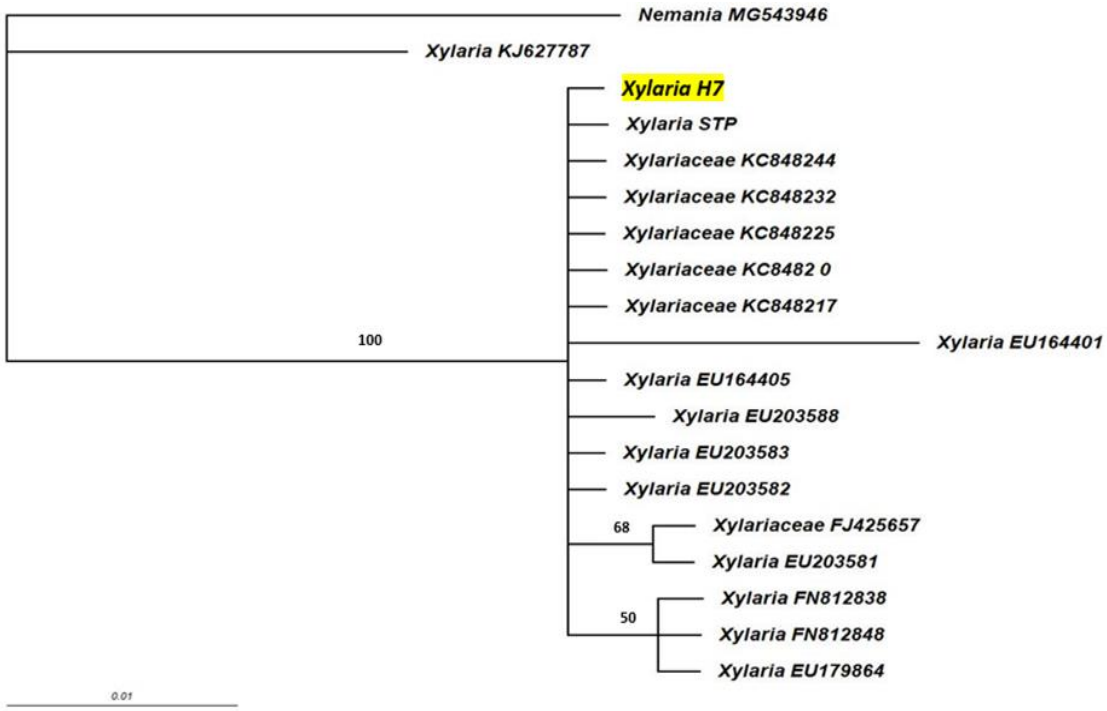

Fig. 1.2 Phylogram prepared using identified fungus sequence (highlighted in yellow).

Note: *Xylaria* STP is a previously identified *Pseudoxylaria* sample obtained from STP mound within IISER Mohali campus.

Microbiome survey

Bacteria belonging to 15 genera and 5 classes (Gammaproteobacteria, Betaproteobacteria, Bacilli, Actinobacteria and Flavobacteria) were obtained and identified from termite and comb samples. Tremendous number of bacteria have been reported to be present in termite mounds. Bacteria obtained from different samples within *Odontotermes obesus* colony is given in the table below.

Culture dependent isolation and identification restrict microbiome survey studies in different ways. Factors like growth media used for culturing, relative abundance of bacteria in samples, competition *etc.*, restrict the range of bacteria which can be obtained using this method. Use of a wide range of growth media can be a possible and partial solution for this problem.

Comb	Worker	Nymph	Alate
Bacillus megaterium	*Acinetobacter baumanni*	*Acinetobacter pitti*	*Acinetobacter calcoaceticus*
Bacillus marsiflavi	*Bacillus BC 152*	*Bacillus aryabhatti*	*Bacillus sp.*
Bacillus pumilus	*Bacillus pumilus*	*Bacillus safensis*	*Citrobacter sp.*
	Bacillus cereus	*Bacillus aquimaris*	*Trabulsiella guamensis*
	Bacillus safensis	*Bacillus FJAT 29894*	*Staphylococcus haemolyticus*
	Cronobacter sp.	*Bacillus pumilus*	*Pseudomonas aeruginosa*
	Citrobacter farmeri	*Cronobacter sp.*	*Paenibacillus sp.*
	Trabulsiella guamensis	*Trabulsiella guamensis*	
	Trabulsiella sp.12	*Pantoea dispersa*	
	Trabulsiella odontotermitis	*Enterobacter sacchari*	
	Burkholderia contaminans		
	Chryseobacterium sp.		
	Microbacterium sp.		
	Serratia rubidaea		
	Enterobacter cloacae		
	Enterobacter sacchari		
	Klebsiella pneumoniae		

Table. 1.1 Bacteria obtained from different samples within *Odontotermes obesus* colony.

CHAPTER 2

Antifungal activity assays

Antifungal activity assays are used to determine the activity shown by certain chemical compounds against fungi. One way of doing it is to determine the zone of inhibition. Zone of inhibition in antifungal activity assays is the area around the source of antifungal compounds in which fungal growth is absent or inhibited. The diameter or area of the zone of inhibition will determine the effectiveness of the antifungal compound, the larger the diameter, greater will be the sensitivity of the fungus to the antifungal compound. Source of antifungal compounds can be filter paper discs with these compounds, bacterial colonies which can produce these compounds or other sources from which the antifungal compounds can diffuse out in the growth media. Antifungal activity assays are used in this study to determine the antifungal activity exhibited by certain bacteria from termite sources against the antagonistic fungus- *Pseudoxylaria.*

2.1 Methods

Potential sources of antifungal compounds used in this study are termite extracts and bacteria obtained from different experiments. PDA was used as the growth media base for the antifungal activity assays. Experimental plates were incubated at 30 °C in dark throughout the experiments.

Extract disc experiments

Freshly collected workers, soldiers and nymphs were washed twice in autoclaved water. Head and abdomen of termites were separated carefully using sterile forceps and needle. Head and abdomen extracts were prepared separately for each termite caste in MCTs by crushing 20 individual parts in 200 µL of 1X PBS. *Pseudoxylaria* plug with PDA base was taken from subculture plates and kept at the center of experimental plate (PDA). Autoclaved filter paper discs were dipped in extracts and placed at 2 sides of the plug on the same day.

Controls:

(a) *Pseudoxylaria* plug only

(b) Extract discs without *Pseudoxylaria* plug

(c) *Pseudoxylaria* plug with filter paper discs dipped in 1X PBS.

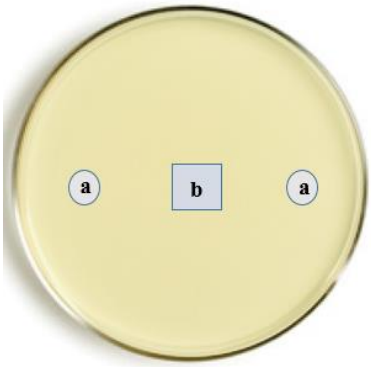

Fig. 2.1 Basic setup for extract disc experiments:

(a) Filter paper discs with extract

(b) *Pseudoxylaria* plug

Extract disc experiment was performed using head and abdomen of workers, soldiers and nymphs. Experimental and control plates were incubated for 5-7 days (by the time which *Pseudoxylaria* grows completely over the PDA plate and starts to die). Plates were observed and images were taken once in every 24 hours.

Bacteria vs *Pseudoxylaria*

Bacterial samples obtained from the control plates of extract disc experiment (extract discs without *Pseudoxylaria*) were isolated and sub-cultured on LB agar plates. Antifungal assays were conducted with each of these bacteria against *Pseudoxylaria.*

Basic experimental setup is same as of extract disc experiment, except a streak or circular inoculate of bacteria were used instead of discs. *Pseudoxylaria* plug was kept at the center of a PDA plate and bacteria were introduced into the plate as streaks or small circular spreads at different sides of the plug. Circular spreads were of same size as the discs used in the previous experiment. Single colony of bacteria was used for both streaking and spreading.

Controls:

(a) *Pseudoxylaria* plug only

(b) Streak/spread without *Pseudoxylaria* plug

Experimental and control plates were incubated for 5-7 days. Plates were observed and images were taken.

2.2 Results and Discussion

Extract disc experiments

Fungal growth was observed in most plates with extract discs of termite samples except the one with worker head extract. Bacteria was observed to be spreading in the plates as a slimy layer. No zone of inhibition was observed. A relative reduction in growth of *Pseudoxylaria* was observed in the experiment in comparison to control *Pseudoxylaria*, suggesting a possible inhibitory effect of bacteria grown from worker head extract discs. As a zone of inhibition was not observed, bacteria were isolated from the plates with extract discs for further experiments.

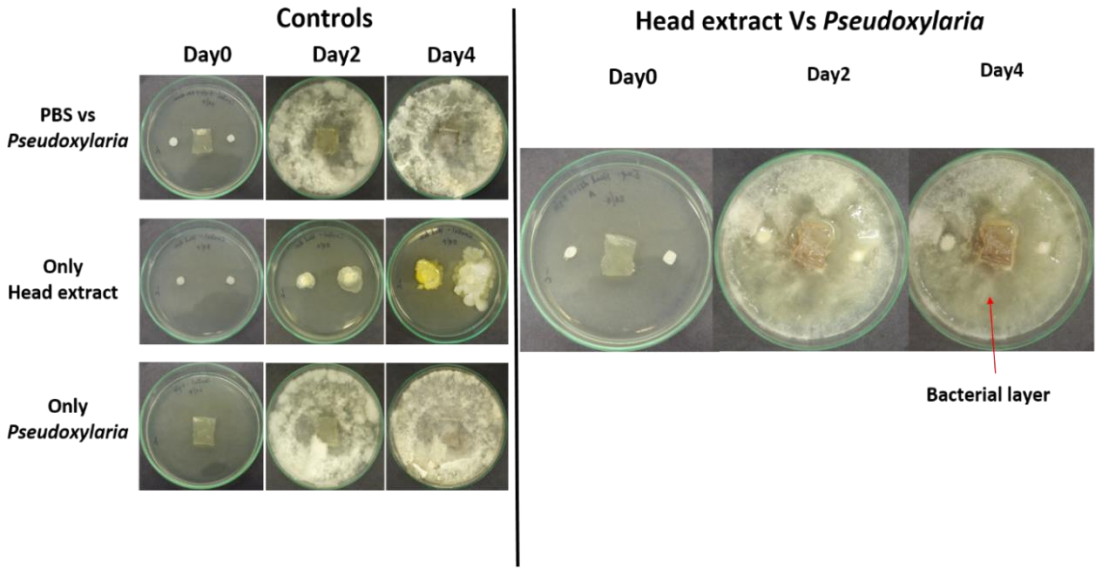

Fig. 2.2 Antifungal activity assay- Extract disc experiment using worker head extract

Biases due to culture dependency may have strong effects on these experiments. As in culture-dependent microbiome survey, factors like growth media used for experiments, relative abundance of bacteria in samples, competition *etc.*, restrict the range of bacteria to be involved in these experiments. These effects may have resulted in the exclusion of potential symbiotic bacteria which can exhibit antifungal properties against *Pseudoxylaria.*

Bacteria vs *Pseudoxylaria*

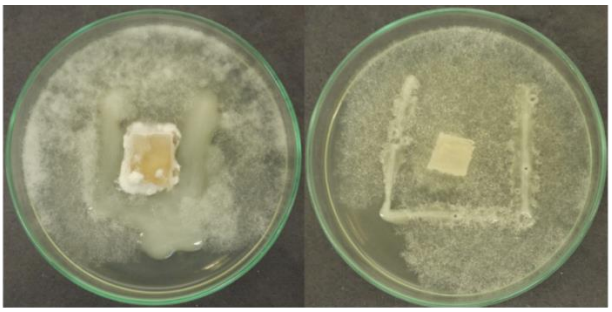

Fig. 2.3 Antifungal activity assay- Streak experiment with *Klebsiella* and *Enterobacter cloacae*

Bacteria obtained from worker head extract experiment were identified as *Enterobacter cloacae* and *Klebsiella pneumoniae. Pseudoxylaria* was observed to grow over streaks of these bacteria in streak experiments and no zone of inhibition was observed.

CHAPTER 3

Response of termites against externally introduced *Pseudoxylaria*

Ability of termites to discriminate between cultivar and antagonistic fungi is crucial in maintaining the monoculture of *Termitomyces* in mounds. It has been demonstrated that workers of the fungus-growing termite *Odontotermes obesus* show discrimination between *Termitomyces* and *Pseudoxylaria* using olfactory cues. Workers were observed to cover the source of olfactory cues produced by antagonistic fungi using soil and agar (Katariya *et al.,* 2017*).* It will be more interesting to look at the behavior of workers when *Termitomyces* monoculture – their food crop faces the threat of invasion from antagonistic fungus *Pseudoxylaria.* If they can protect their fungus combs from getting infected by antagonistic fungi, it is important to understand the mechanism behind the process. If they can suppress the initiation of growth of *Pseudoxylaria,* it is likely that they can inhibit the spreading of grown mycelia also. Experiments were designed in order to create situations in which the termites are forced to employ their defense mechanisms to protect their monoculture in the presence of externally introduced *Pseudoxylaria*. This increases the probability of finding agents mediating this process from a biologically relevant context.

3.1 Methods

Fresh fungus combs, workers and soil were collected from the mound. *Pseudoxylaria* obtained from 3 days old comb was sub-cultured regularly.

Experimental setup

Glass petri-dish with 20 grams of autoclaved mound soil was used as the base. Fresh comb piece (1.5 grams) with *Termitomyces* nodules was kept at the center of the plate with 30 workers. Mycelia of sub-cultured *Pseudoxylaria* (0.05 grams) and pieces of

Pseudoxylaria infected comb (0.5 grams) was kept in the plate. 3 mL of autoclaved water was provided into this system.

Controls:

(a) Base with fresh comb only.

(b) Base with fresh comb and workers without external *Pseudoxylaria.*

Experimental setup was incubated in dark at 28 °C and 85% Relative humidity. Plates were monitored and images were taken once in every 12 hours for first 2 days of experiment and once in 24 hours for rest of the days. Experiments were continued until most of the workers were dead after the comb with *Termitomyces* got exhausted.

Idea behind this experiment was to observe the behavior of workers in the presence of fresh comb on the introduction of external *Pseudoxylaria* (mycelia) into the termite-fresh comb system. How the workers deal with this scenario is important in finding the situations in which the abundance of potential defensive bacterial symbiont candidates is relatively high.

3.2 Results and discussion

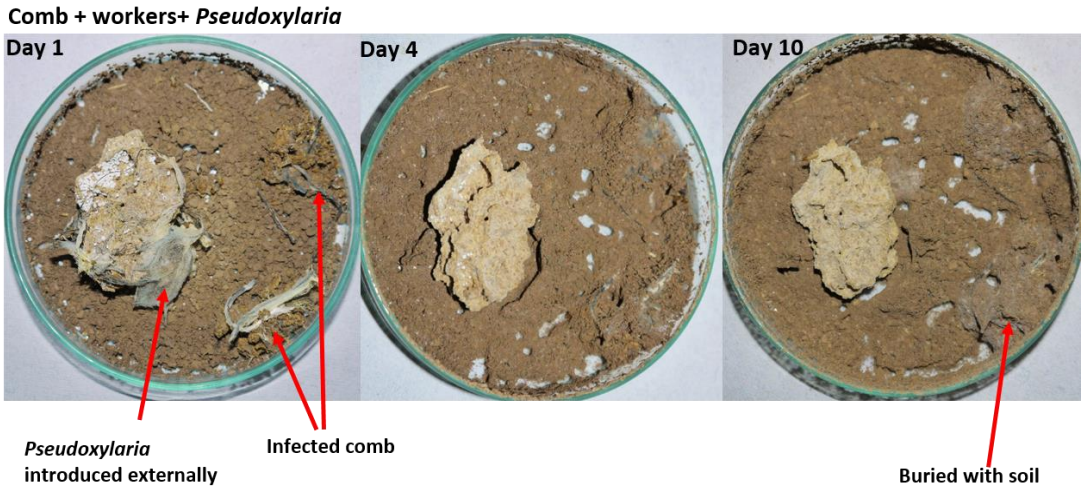

Fig. 3.1 Response of termites against externally introduced *Pseudoxylaria*- Experimental plates

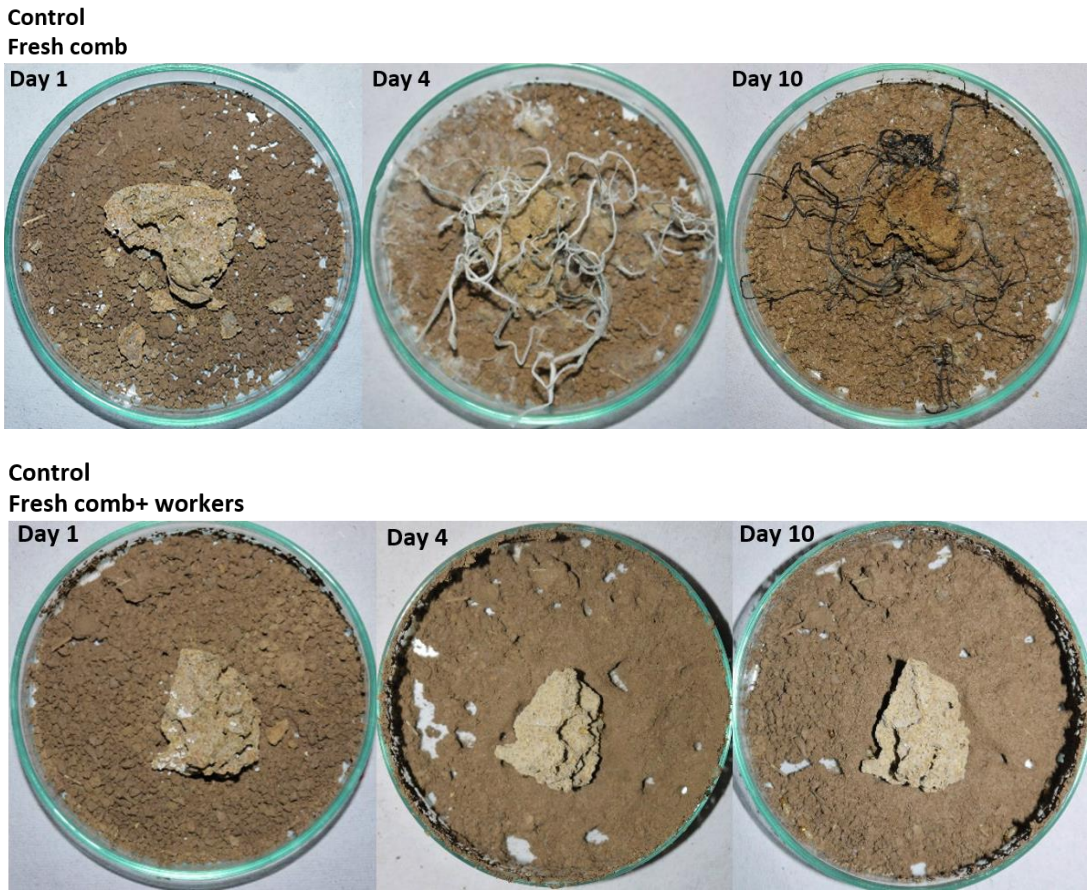

Fig. 3.2 Response of termites against externally introduced *Pseudoxylaria*- control plates

Initiation of growth of *Pseudoxylaria* on fungus comb was observed in control plates without termites on day 2. In experimental plates, it was observed that *Pseudoxylaria* mycelia kept on the fresh comb was removed during first 12 hours of the experiment. Externally introduced infected comb and mycelia of *Pseudoxylaria* was found to be getting covered with soil in first 24 hours and were completely covered with soil in 4-5 days. Growth of *Pseudoxylaria* was not observed after they were completely covered in soil. Fresh combs with *Termitomyces* were found intact and devoid of *Pseudoxylaria* infection until most of the workers were dead (observed also in control plates with termites, without external *Pseudoxylaria*). Externally introduced infected comb and mycelia of *Pseudoxylaria* were observed to be prevented from spreading on the soil and onto the fresh comb by the means of this soil covering mechanism, suggesting the presence of potential antifungal compounds in this soil. Soil by which *Pseudoxylaria* was covered was found to be harder than the base soil, which suggests that the termites might be mixing the soil with oral secretions before covering. If there are any bacterial

symbionts involved in this burying process, it is likely that the termites are employing these symbionts through soil.

The soil which termites used to bury *Pseudoxylaria* and infected comb was collected, mixed with autoclaved water and cultured on LB agar and PDA plates. Colonies were isolated and these bacteria were identified as *Serratia* and *Klebsiella pneumonia. Serratia* was used to perform antifungal activity assay against *Pseudoxylaria* using streak method.

Klebsiella pneumonia showed negative results in antifungal activity assay performed in previous experiments. Zone of inhibition was not observed in antifungal activity assays performed using *Serratia.*

All antifungal activity assays performed using bacteria obtained from termite samples and experiments showed negative results. None of these bacteria were found to have the ability to inhibit the growth of *Pseudoxylaria* in co-culture. It is important to widen the range of growth media to increase the number of cultivable bacteria which can be obtained from different experiments. Increasing the sample size in this way will increase the probability of finding bacteria with expected properties.

Another way to find potential symbiotic bacteria with antifungal activity against *Pseudoxylaria* is to adopt culture-independent methods. Soil samples can be collected from the behavioral experiment in a daily manner and DNA isolation can be performed. This DNA can be used to perform Next Generation Sequencing techniques in order to observe the difference in relative abundance of bacteria present in these samples. Comparing this data from soil used to bury *Pseudoxylaria* with that of background soil, along with the status of *Pseudoxylaria* buried in soil may provide useful information about bacteria with desired properties. Potential symbiotic bacteria used by termites to inhibit *Pseudoxylaria* are expected to be relatively abundant in the soil used by termites to bury *Pseudoxylaria* than in the background soil, and this relative abundance is expected to decrease after the complete suppression of *Pseudoxylaria.*

Previous studies in fungus growing termites have reported the presence of potential symbiotic bacteria which produces compounds with antifungal activity against antagonistic fungi (Mathew *et al.,* 2011; Um *et al.,* 2013). But none of them could prove that the termites are using these bacteria in selectively inhibiting antagonistic fungi found in termite colonies. Further studies have to be conducted to isolate bacteria with

desired properties from biologically relevant situations (like the scenario in behavioral experiments conducted in this study) to understand more about the role of symbiotic bacteria in the process of maintaining the monoculture of *Termitomyces* in termite colonies.

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