nature portfolio

Peer Review File

Investigation into how Odontotermes obesus maintains a predominantly Termitomyces monoculture in their fungus combs suggests a potential partnership with both fungi and bacteria

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Assessing how termites prevent pathogens is an interesting topic. This manuscript tried to describe that Odontotermes obesus harbored a vast of microbes which could protect O. obesus fungus gardens against fungal invaders by microbiota analysis and in vitro anti-fungal assays. However, there are several major concerns regarding this work:
1. The combs were collected from only two sites, and the samples are not enough. The authors should collect the samples from different places.
2. The rarefaction curves were not smooth in Figure S7 these indicated that the sequencing depth were not enough.
3. The activities of the tested bacterial mutualists against the fungal contaminants were weakly presented in Figure 4.
4. In general, the manuscript lacks novelty.
Thus, the manuscript was not acceptable for publication in Communications Biology.

Reviewer #2 (Remarks to the Author):

Review of “Partnership with both fungi and bacteria can protect Odontotermes obesus fungus gardens against fungal invaders” by Agarwal et al.

This manuscript addresses the way how fungus-growing termites can maintain cultures of symbiotic fungi largely pathogen and weed free. Fungus-growing termites cultivate fungi of the genus Termitomyces on combs composed of dead plant material collected by foraging workers in the colony environment. It is unknown how termites can maintain their colonies pathogen free. The present manuscript addresses this question by assessing mycobiomes and microbiomes from various sources of a colony of Odontotermes obesus, including workers, alates and combs with various periods of incubations in the absence of termites. The overall quality of the manuscript seems good and the results are interesting. I do have some comments and suggestions for improvement, however, which I outline below.

My main comment is on the interpretation of the competition assays. For Termitomyces and Pseudoxylaria, you use different methods to inoculate them to test competitive strength of various other fungi. I can understand that the authors have done this, as this reflects differences in the biology of these two fungi. However, I think it should be explained in more detail, and also why the authors have chosen 36 hours of pre-incubation of Termitomyces. The pre-incubation time, and the inoculation density, are critical for the competitiveness of Termitomyces and this should be discussed, and preferably also an indication of the inoculation density. However, given this choice of 36 hours and this density, the relative competitiveness of Termitomyces against other fungi can be measured, but it should be made clear that it is not obvious how this translates to a real colony when Termitomyces has either not had a chance to pre-grow before a fungus is introduced, or has

had a longer time of pre-growth. Similar background needs to be provided for the relevance of the Pseudoxylaria assays.

Page 3 lines 36-37: The authors speak of lower and higher termites, but it has recently been proposed to abandon the terms higher and lower, since they suggest an unwarranted direction in evolution ("A call to termitologists: it is time to abandon the use of "lower" and "higher" termites" Carrijo et al Insectes Sociaux 2023). I think it will be good to follow their suggestions and to use alternative terms.

Line 41: Termitomyces is singular so use utilizes. Please check throughout the manuscript.

Line 54: "of other non-desirable fungi". Please add: a comma, to get "of other, non-desirable fungi".

Line 78: "Another evidence" change to "Another line of evidence"

Line 81: "and to what extent can Termitomyces act against these non-specific fungi". Change to "and to what extent Termitomyces can act against these non-specific fungi"

Line 128: change "least" to "lowest".
Figure 2: It is unclear how to interpret the figures. First, the size of the circles indicates abundance, similar to the y axis values? Where does the ~10% non-specific fungi at 24 hours come from, and 99.9% after 96 hours? There is still a significant red circle at 96 hours. Please explain.
And how can I interpret the different results obtained by qPCR (Figure 2B) and nanopore sequencing (2A)? Please provide an explanation.

Furthermore, how do those percentages of Termitomyces relate to the pie chart depicted in Figure 1?

Figure 3 indicates that most fungi were equally inhibited by Pseudoxylaria and Termitomyces, and the text (l208-214) indicates that there were two exceptions, where a fungus was inhibited by one of the two only. However, Trichoderma is not mentioned, and according to figure 3 it is inhibited by Pseudoxylaria but not by Termitomyces. Please clarify.

Lines 215-220. "The eight bacterial mutualists, from four different genera, showed inhibitory effects against all but one (Fusarium-MN913755) of these fungi (Fig. 4, Table 2)." This sentence is a bit confusing, as it suggests at first sight that all eight bacterial mutualists show inhibition, while what is meant is that 7 of the eight fungi are affected by at least one of the bacteria. Please re-formulate.

Lines 248-250: "However, as Supplementary figure S11 illustrates, the presence of both Termitomyces and Pseudoxylaria is correlated with the contaminating fungi being in check for at least the first 48 hrs." To me the figure shows that this is the case for the first 24 hours as time point 48 hours already shows a dominance of other fungi.

If Pseudoxylaria plays a role in suppressing other fungi, what remains enigmatic is that it has such

low abundance when the colony is alive. It only starts to emerge when the termites are gone. Please discuss this.

Line 272-274: You indicate that Termitomyces declines after 48 hours. I think it is reasonable to assume that this is mostly a decline, relative to Pseudoxylaria, rather than an absolute decline. Please discuss.

Line 301: change “show” to “shows”

Reviewer #3 (Remarks to the Author):

Review of manuscript COMMSBIO-23-2987-T

This manuscript claims that fungus-growing termite combs harbors a diverse microbial community of bacteria and fungi, in addition to Termitomyces fungal cultivars. It further argues on how Pseudoxylaria and Termitomyces interact to suppress other fungi potentially harmful to fungus combs. Evidence from plate assays, some bacterial symbionts along with Termitomyces and Pseudoxylaria secrete antifungal that keep fungi in check to proliferate in the combs.

The results are novel as this is the first study to empirically explain how the microbiota of the fungus combs may help termites grow monocultures of Termitomyces in the presence of the so-called “weed fungi”. This is of interest for the community of researchers that study fungus-growing social insects. I congratulate the authors for performing the fungus comb decay experiments and come up with a nice interpretation of the dynamics of the comb mycobiota.

Although I feel important insights emerge from this study, my major concern is on the strengthen of these insights and how they impact our understanding of Pseudoxylaria. Figure 5 brings the main message of the manuscript, but the hypothesis is based on sampling of only two colonies. To circumvent this issue, I suggest carrying out qPCR assays on decaying combs of additional colonies to support the findings. In addition, one way to test whether Pseudoxylaria suppress additional fungi to overgrow combs is finding termite colonies that are Pseudoxylaria-free and conduct the decay experiments followed by qPCR profiling. If weed fungi overgrow decaying combs in different abundances in the absence of Pseudoxylaria, this would be compelling evidence for a possible mutualistic role of this fungus. On a side note, the manuscript does not mention whether there are termite colonies free of Pseudoxylaria.

Additional comments:

L. 14: Why should one consider every fungus entering combs a pathogen? Perhaps, the terminology is the issue: a fungus can be a threat to combs in the sense it is a weed, but this does not necessarily means it is a pathogen that causes disease in combs. I agree that some fungi can be a pathogenic (or even a parasite) to combs, but label all as pathogenic seems not representative of what they really are. This discussion is important, as the whole manuscript assumes a “pathogenic

load" (L. 16, 89-90 and many others). If one needs to label what these fungi are doing in combs, I would say the results of the present study support that they act as opportunists. This would be a more reasonable lifestyle for these fungi to consider.

L. 23: A hypothesis (Figure 5) derived from in vitro plate experiments seems fragile as we do not have evidence if the interactions (and gene expression) in vitro really mirror what happen in combs. Please, discuss how representative are these experiments for fresh combs.

L. 26: I believe there is a distinction here regarding concepts: (i) monoculture: this is related to fungi used for food (ii) and microbe-free combs: this is related to other organisms associated with the comb but not the cultivar. Fungus-growing insects can grow monocultures, even so in the presence of not microbe-free comb. I would consider carefully choosing between these concepts and be consist throughout the manuscript.

L. 34 and 39: replace to "basidiomycete".

L. 41: I believe it is "utilizes".

L. 57: change to "saprotrophic" as saprophytic are more related to plants.

L. 118-119: The 0h termite-free combs also have 111 fungal genera (Table 1). It seems quite a lot just to say they are not growing in fresh combs. How indicative is it to consider these fungi are not actively growing in fresh combs? This is also a major issue that is not clear in the manuscript.

L. 124: I really miss figures with the termite workers, general nest structure and fungus combs to illustrate the study system. Perhaps, bring some of the beautiful photos in the supplementary material to the main manuscript.

L. 224: "fungal genera" instead of "fungal strains" according to Table 1?

L. 241: I believe also Termitomyces and bacteria as shown in this study, right?

L. 472-473: Why make this selection? Would it make more sense to have used all the other fungal contaminants against the bacteria too?

Figure 1: There are no numbers under the pie charts. Also, are the genera highlighted in blue or in bold?

Figure 2: Is Pseudoxylaria present in all termite nests in a population? To test the hypothesis that Pseudoxylaria is competing with other fungi, instead of in vitro interactions would be more interesting to evaluate the dynamics in combs were Pseudoxylaria is not present in all stages of decay? I believe this would be the treatment to test the hypothesis.

Figure 3: ITS is not a gene.

Figure 5: Name “bacterial mutualists” means that bacteria are benefiting from the association. Is this true for all bacteria examined in the present study? Do we know the reciprocal benefits of bacteria and Termitomyces for all bacteria found in combs. I believe it is better to specify which bacteria this manuscript is building the hypothesis.

Table 1: The manuscript will read better if the decay stage 0h, which is really not a decay treatment yet, could be renamed to “fresh comb”.

Table 2: This table could go in the supplementary since Figure 4 summarizes the results already.

Reviewer 1, comment 1: The combs were collected from only two sites, and the samples are not enough. The authors should collect the samples from different places.

Reviewer 3, comment 1: Although I feel important insights emerge from this study, my major concern is on the strengthen of these insights and how they impact our understanding of *Pseudoxylaria*. Figure 5 brings the main message of the manuscript, but the hypothesis is based on sampling of only two colonies. To circumvent this issue, I suggest carrying out qPCR assays on decaying combs of additional colonies to support the findings.

Response 1: Unfortunately, there are several problems in getting additional samples. First, the amounts of combs sampled in our study cannot be sustainably done with every mound. We have taken around 1-1.5 kg of comb samples from these two mounds. Therefore, we need very large, mature and relatively older termite mounds which are very rare. Our location is right in the middle of three cities (Chandigarh-Mohali-Panchkula Tricity) with a combined population of over 2.6 million people and it is difficult to find such mounds nearby which are relatively undisturbed. We were planning to sample more mounds starting from the plains to across the Himalayan foothills. However, heavy unseasonal rainfall in March of 2023 (https://weather.com/en-IN/india/news/news/2023-03-21-unseasonal-rains-flight-diversions-in-delhi-crop-damage-in-up-punjab) has severely hampered our plans to do so. Some of the mounds in our vicinity, including the two mounds used in the study, have suffered extensive damage as they were under water for several days. Extracting large volumes of fungus combs from these already distressed mounds, we fear, will not allow them to continue further. Their sustainability is key as, in addition to being termite researchers, we are also custodians of these mounds. Second, the first and the second authors have now left the lab for post-doctoral positions and there is a dearth of trained people to do this work.

We would request the reviewers to please consider the following:

a. These two mounds are the basis of two studies, Agarwal et al 2021 and the current one. These two studies have detailed the pan-microbiota (Agarwal et al 2021) and the pan-mycobiota (current study) resulting in one of the most extensive microbial data sets available on any fungus-growing termites.
b. We agree that the combs were collected only from two mounds but we have gone for depth of sampling and collected samples from these two mounds consecutively for three months.
c. We have sampled 12 pieces of fresh fungus comb, 30 termites across different castes and 36 samples of the decaying comb across various stages for Nanopore sequencing. For qPCR analysis, 72 samples of degrading comb (12 for each stage) were used to quantify the absolute abundances of *Termitomyces*,

Pseudoxylaria and *Pseudomonas*. Moreover, each of these samples were run as a triplicate for the qPCR assays. This extensive sampling of these mounds has now enabled us to have a far better understanding of how the microbes within a termite mound functions.

In addition to these lines (Now line 336-338) we have added the following:

This model, however, awaits empirical validation, and future studies with more mounds and different species of termites are needed to establish whether this model is validated or the role of *Pseudoxylaria* remains that of a weed and/or parasite.

2. **Editor's comment:** -Additional sequencing data should be generated to address **Reviewer #1's** concern regarding insufficient sequencing depth.

Reviewer 1, comment 2: The rarefaction curves were not smooth in Figure S7 these indicated that the sequencing depth were not enough.

Response 2: We realize that the rarefaction curves do not flatten out. This is in spite of our efforts of multiple sequencing reactions of the same data (especially for Minor workers and Nymphs). However, what is more important is what these curves represent. One of our aims was to uncover the number of fungal and bacterial genera in these termite castes and combs. Our estimates, detailed in figure 2, figure S6, table S9 and table S10 show more of these than any other studies on these termites.

We present a table of the number of reads obtained from different studies compared with the present one. As is clear from this, the number of reads that our studies have generated is still the highest. Therefore, this study represents a far more detailed estimate of the microbes present in any fungus-growing termite till date.

Fungal genera obtained from this and other studies on fungus-growing termites

Termite sample	Sample	Method of sequencing	Number of reads generated	Number of different fungal genera found	Reference

Macrotermes natalensis 115	Fungus comb	454 Pyrosequencing	0.36×10^5	1	Otani et al., 2019
Macrotermes natalensis 116			0.53×10^5	1	
Macrotermes natalensis 118			0.41×10^5	1	
Macrotermes natalensis 132			0.24×10^5	1	
Macrotermes natalensis 134			0.33×10^5	1	
Macrotermes natalensis 135			0.40×10^5	1	
Macrotermes natalensis 136			0.51×10^5	1	
Macrotermes natalensis 141			0.30×10^5	1	
Odontotermes badius 111			0.41×10^5	1	
Odontotermes badius 112			0.42×10^5	1	
Odontotermes badius 114			0.33×10^5	1	
Odontotermes badius 122			0.21×10^5	1	
Odontotermes badius 126			0.12×10^5	1	
Odontotermes.sp 120			0.24×10^5	1	

Odontotermes.sp 121			0.35×10^5	1	
Odontotermes.sp 124			0.51×10^5	2	
Odontotermes.sp 125			0.47×10^5	2	
Odontotermes.sp 127			0.36×10^5	1	
Odontotermes.sp 143			0.43×10^5	1	
Macrotermes natalensis 115	Fungus comb	Illumina MiSeq	0.17×10^5	4	Otani et al., 2019
Macrotermes natalensis 116			0.15×10^5	3	
Macrotermes natalensis 118			0.05×10^5	6	
Macrotermes natalensis 132			0.10×10^5	2	
Macrotermes natalensis 134			0.26×10^5	3	
Macrotermes natalensis 135			0.26×10^5	7	
Macrotermes natalensis 136			0.22×10^5	5	
Macrotermes natalensis 141			0.09×10^5	1	
Odontotermes badius 111			0.20×10^5	7	

Odontotermes badius 112			0.25×10^5	6	
Odontotermes badius 114			0.35×10^5	4	
Odontotermes badius 122			0.25×10^5	5	
Odontotermes badius 126			0.14×10^5	1	
Odontotermes.sp 120			0.26×10^5	6	
Odontotermes.sp 121			0.13×10^5	5	
Odontotermes.sp 124			0.25×10^5	8	
Odontotermes.sp 125			0.55×10^5	6	
Odontotermes.sp 127			0.23×10^5	2	
Odontotermes.sp 143			0.10×10^5	1	
Macrotermes bellicosus	Gut	Illumina	2.7×10^5	51	Bos et al., 2020
Macrotermes barneyi	Gut	Culturing	N/A	9	Nagam et al., 2021
	Fungus Comb		N/A	13	
Odontotermes obesus		Nanopore Sequencing		**Total 61**	This study
	Worker		0.8×10^5	46	
	Nymph		0.17×10^5	41	
	Alates		1.18×10^5	43	
	Fungus comb (0 hrs)		0.54×10^5	31	

	Fungus comb (24 hrs)		$0.58x10^5$	33	
	Fungus comb (48 hrs)		$0.14x10^5$	42	
	Fungus comb (72 hrs)		$0.14x10^5$	41	
	Fungus comb (96 hrs)		$0.15x10^5$	38	
	Fungus comb (120 hrs)		$0.04x10^5$	34	

Bacterial genera obtained from different studies on fungus growing termites

Termite sample	Sample	Method of sequencing	No. of reads generated	Number of different bacterial genera	Reference
Odontotermes sp.	Gut	Pyrosequencing	$1.12*10^5$	112	Dietrich et al., 2014
Macrotermes subhyalinus	Gut		$0.27*10^5$	205	
Ancistrotermes guineensis	Gut	Pyrosequencing	$0.09*10^5$	59	Otani et al., 2014
Macrotermes subhyalinus	Gut		$0.24*10^5$	108	
Microtermes toumodiensis	Gut		$0.22*10^5$	71	
Odontotermes sp3	Gut		$1.18*10^5$	85	
Pseudacanthotermes militaris	Gut		$0.24*10^5$	88	

Macrotermes natalensis 115	Comb	Pyrosequencing	$0.12*10^5$	81	Otani et al., 2016
Microtermes sp. 127	Comb		$0.20*10^5$	196	
Odontotermes cf. badius 112	Comb		$0.26*10^5$	177	
Macrotermes natalensis 134JS1	Gut	Illuminas Miseq	$1.13*10^5$	58	Otani et al., 2019
Macrotermes natalensis 134JW3	Gut		$0.17*10^5$	79	
Macrotermes natalensis 134NS1	Gut		$0.17*10^5$	51	
Macrotermes natalensis 134NW1	Gut		$0.17*10^5$	83	
Odontotermes 129NW1	Gut		$0.47*10^5$	47	
Odontotermes 143JW1	Gut		$0.46*10^5$	52	
Odontotermes 143S2	Gut		$0.33*10^5$	40	
Ancistrotermes_guineensis_K	Gut		$0.13*10^5$	26	
Ancistrotermes_guineensis_Q	Gut		$0.12*10^5$	12	
Macrotermes natalensis_137_K_1	Gut		$0.12*10^5$	13	
Macrotermes natalensis_156_Q_3	Gut		$0.17*10^5$	12	
Odontotermes_sp_K_3	Gut		$0.08*10^5$	14	
Odontotermes_sp_Q_1	Gut		$0.09*10^5$	20	
Pseudocanthotermes_militaris_K_2	Gut		$0.15*10^5$	11	
Pseudocanthotermes_militaris_Q_2	Gut		$0.12*10^5$	9	

Odontotermes formosanus	Fresh Fungus comb	Illumina MiSeq	$1.29*10^5$	47	Ahmad et al., 2022
	Mature fungus comb		$1.39*10^5$	47	
	Old comb		$1.39*10^5$	39	
	Old Worker		$1.16*10^5$	44	
	Young worker		$1.29*10^5$	40	
Odontotermes obesus	Major Worker	Nanopore Sequencing	$0.8*10^5$	506	Agarwal et al., 2021
	Minor worker		$0.03*10^5$	127	
	Nymph		$0.10*10^5$	140	
	Male		$1.11*10^5$	335	
	Female		$0.27*10^5$	262	
Odontotermes obesus	Fresh fungus comb (0 hrs)	Nanopore Sequencing	$0.54*10^5$	405	This study
	Fungus comb (24 hrs)		$0.58*10^5$	260	

	Fungus comb (48 hrs)		$0.14*10^5$	1355	
	Fungus comb (72 hrs)		$0.14*10^5$	254	
	Fungus comb (96 hrs)		$0.15*10^5$	199	
	Fungus comb (120 hrs)		$0.04*10^5$	193	

3. **Editor's comment:** -From an editorial perspective and in light of **Reviewer #1's** concern that your manuscript appears to lack novelty, please clearly describe in your revised manuscript how this study builds on your previous work in reference #33.

Reviewer 1, comment 4: In general, the manuscript lacks novelty.

Response #3: We are very surprised by this statement as, to the best of our knowledge, this study is the first to:

1. Identify the pan-mycobiota of any fungus-growing termite including those of alates.
2. Identify the micro and mycobiota of a decaying fungus comb.
3. Verify *Termitomyces* and *Pseudoxylaria* reads by using qPCR in healthy and decaying combs.
4. Verify the dynamics of potential bacterial mutualist with respect to *Termitomyces* and *Pseudoxylaria* using qPCR.
5. Test the roles of bacterial mutualists in preventing non-specific fungi other than *Pseudoxylaria*.
6. Test the role of *Pseudoxylaria* against other non-specific fungi and propose its role as a symbiont.

This study substantially differs from our previous study by Agarwal et al., (2021), where we identified the pan-microbiota, and not mycobiota, from these same mounds. Moreover, we showed, for the first time, the role of *Pseudomonas* and *Burkholderia* as potential bacterial mutualists in any fungus-growing termite. We also confirmed the role of previously known candidate mutualists *Bacillus* and *Streptomyces* from fungus-growing termites.

Below, we present a detailed response to the other comments from the reviewers.

Reviewer #1

Reviewer 1, comment 3: The activities of the tested bacterial mutualists against the fungal contaminants were weakly presented in Figure 4.

Response: Without a proper explanation as to why Fig 4 (now updated to Fig. 5) is 'weak', it is difficult for us to make specific corrections. The organization of the figure is similar to what we have published before. It summarizes the interactions of various bacteria and fungi found from these mounds. Moreover, the detailed data is also presented in the supplementary materials as figures S15-S22. We understand that representation of such complex data can be difficult and we will make improvements if the reviewer has any specific suggestions for the figure.

Reviewer #2

Reviewer 2, comment 1: My main comment is on the interpretation of the competition assays. For *Termitomyces* and *Pseudoxylaria*, you use different methods to inoculate them to test competitive strength of various other fungi. I can understand that the authors have done this, as this reflects differences in the biology of these two fungi. However, I think it should be explained in more detail, and also why the authors have chosen 36 hours of pre-incubation of *Termitomyces*. The pre-incubation time, and the inoculation density, are critical for the competitiveness of *Termitomyces* and this should be discussed, and preferably also an indication of the inoculation density. However, given this choice of 36 hours and this density, the relative competitiveness of *Termitomyces* against other fungi can be measured, but it should be made clear that it is not obvious how this translates to a real colony when *Termitomyces* has either not had a chance to pre-grow before a fungus is introduced, or has had a longer time of pre-growth. Similar background needs to be provided for the relevance of the *Pseudoxylaria* assays.

Response: Our experience in growing *Termitomyces* and *Pseudoxylaria* on PDA plates indicates that the former grows much more slowly than the latter. While *Pseudoxylaria* plugs can fill up a control Petri plate within 5 days, *Termitomyces* plugs take over 30 days to do so (Figure now in supplementary S11). This is not a unique observation as some previous studies (Botha and Eicker, 1991) have also reported this. Consequently, several different measures were also taken to test the effectiveness of *Termitomyces* against some fungi, including *Pseudoxylaria*, like flipping the plates, which had previously grown *Termitomyces*, and checking the residual inhibitory effects against other fungi (Otani et al., 2019). We have taken a different approach which was detailed in Agarwal et al (2021). Due to constraints on word limits we referred to the same study and did not elaborate further. However, we have remedied this in lines 462-470. Briefly, to account for the growth disparity, a liquid suspension of *Termitomyces* nodules and mycelia was prepared by homogenizing a 4 cm^2 blocks of actively growing culture (10-12 days old) in sterile 1X PBS and then spread it on plates with Potato-Dextrose with Yeast Malt (PYME) Agar.

The pre-incubation time of 36 hours was selected on the basis of our pilot experiments (figure S10 now in supplementary). In these pilot assays *Termitomyces* was grown for both 24 hrs and 36 hrs before the introduction of the competing microbes. We found no differences in the results after 7 days of incubation and therefore selected a pre-incubation time of 36 hrs. Moreover, in a fresh fungus comb *Termitomyces* dominates. Therefore, we wanted to mimic this feature of the fresh fungus comb in our *in vitro* plates, as far as possible, and tried covering the entire plate with *Termitomyces* culture, before the introduction of the test fungus. However, we agree with the reviewer that we are still unclear how such inhibition takes place when *Termitomyces* is not yet established, like the beginning of a new comb or founding of a new colony. Current research in the lab is trying to answer some of these questions.

Reviewer 2, comment 2: Page 3 lines 36-37: The authors speak of lower and higher termites, but it has recently been proposed to abandon the terms higher and lower, since they suggest an unwarranted direction in evolution ("A call to termitologists: it is time to abandon the use of "lower" and "higher" termites" Carrijo et al Insectes Sociaux 2023). I think it will be good to follow their suggestions and to use alternative terms.

Response: We have corrected this. We have now replaced higher termites with "Termitidae" and lower termites with "non-Termitidae termites" (Line 37-40).

Reviewer 2, comment 3: Line 41: Termitomyces is singular so use utilizes. Please check throughout the manuscript.

Response: We have made the corrections in Line 43 and also across the manuscript.

Reviewer 2, comment 4: Line 54: “of other non-desirable fungi”. Please add: a comma, to get “of other, non-desirable fungi”.

Response: We have now added the comma (Line 55).

Reviewer 2, comment 5: Line 78: “Another evidence” change to “Another line of evidence”

Response: We have changed Another evidence to ‘Another line of evidence’ (Line 79-80).

Reviewer 2, comment 6: Line 81: “and to what extent can Termitomyces act against these non-specific fungi”. Change to “and to what extent Termitomyces can act against these non-specific fungi”.

Response: We have now changed the statement (Line 82-83).

Reviewer 2, comment 7: Line 128: change “least” to “lowest”.

Response: We have changed “least” to “lowest” (Line 130).

Reviewer 2, comment 8: Figure 2: It is unclear how to interpret the figures. First, the size of the circles indicates abundance, similar to the y axis values? Where does the ~10% non-specific fungi at 24 hours come from, and 99.9% after 96 hours? There is still a significant red circle at 96 hours. Please explain. And how can I interpret the different results obtained by qPCR (Figure 2B) and nanopore sequencing (2A)? Please provide an explanation.

Response: There was an error in the labels which we have now fixed. The Nanopore data is represented in the second panel of figure 3 (as figure 3b; figure 2 is now updated to figure 3) and the qPCR data is represented in the bottom panel, as box plot, in figure 3c.

In Figure 3b, the sizes of the circles indicate abundance, which is represented in the y axis. However, the plot includes only the fungi with a relative abundance of at least 0.3%. There are several other non-specific fungi that were found at 0 hrs (fresh comb) and also during the course of the degradation. However, representing all of them would have made the figure extremely cluttered and, we fear, unintelligible. However, the entire data is presented in Supplementary table S9. The red circles in figure 3 represents *Pseudoxylaria*, which reached a maximum at 48 hrs (26%) and continued to be present with a slightly lower abundance till the end of degradation (<12%).

Figure 3c represents the number of copies of *Termitomyces* and *Pseudoxylaria* as obtained through qPCR analysis and indicates the similar pattern of dynamics, although differing in magnitude, with the Nanopore data. The green box represents the number of copies of *Termitomyces,* which is highest till 24 hrs, and drops drastically after that. The red box represents the number of copies of *Pseudoxylaria* which reached the maximum relative abundance at 48 hrs.

Reviewer 2, comment 9: Furthermore, how do those percentages of *Termitomyces* relate to the pie chart depicted in Figure 1?

Response: The heatmap in figure 2 (figure 1 updated to figure 2) represents the nanopore data obtained from all the castes and degrading fungus comb. The pie chart below the heatmap shows the abundance of *Termitomyces* and all other fungi in that data. We have now included the percentages below the pie chart for all the sample types.

Reviewer 2, comment 10: Figure 3 indicates that most fungi were equally inhibited by Pseudoxylaria and Termitomyces, and the text (l208-214) indicates that there were two exceptions, where a fungus was inhibited by one of the two only. However, Trichoderma is not mentioned, and according to figure 3 it is inhibited by Pseudoxylaria but not by Termitomyces. Please clarify.

Response: We have now updated the line 211-213 to read:

The exceptions are *Mucor*- MN913757 and *Trichoderma*- MN913767 where the major inhibition is shown by *Pseudoxylaria* whereas *Phialotubus* -MN913760 is inhibited more by *Termitomyces.*

Reviewer 2, comment 11: Lines 215-220. "The eight bacterial mutualists, from four different genera, showed inhibitory effects against all but one (Fusarium-MN913755) of these fungi (Fig. 4, Table 2)." This sentence is a bit confusing, as it suggests at first sight that all eight bacterial mutualists show inhibition, while what is meant is that 7 of the eight fungi are affected by at least one of the bacteria. Please re-formulate.

Response: We have rewritten the sentence in lines 217-220 as:

"Seven of the eight fungal contaminants were inhibited by at least one of the bacterial mutualists. *Fusarium*-MN913755 was not inhibited by any of the bacterial strains (Fig. 5 and Supplementary figs. S15-S22). As figure 5 indicates, out of the eight bacterial strains, six showed a significant capability to prevent the growth of some of these fungi (Table S8)."

Reviewer 2, comment 12: Lines 248-250: "However, as Supplementary figure S11 illustrates, the presence of both Termitomyces and Pseudoxylaria is correlated with the contaminating fungi being in check for at least the first 48 hrs." To me the figure shows that this is the case for the first 24 hours as time point 48 hours already shows a dominance of other fungi.

Response: The relative abundance does show a marked decrease in *Termitomyces* by 24 hrs. However, the absolute abundance data, by qPCR, shows presence of *Termitomyces* in appreciable numbers even after 48 hrs of incubation. That is why we had written the statement. However, we agree with the reviewer's suggestion. We have updated the statement to (Line 252-254):

"However, as Supplementary figure S8 illustrates, the presence of both *Termitomyces* and *Pseudoxylaria* is correlated with the contaminating fungi being in check for at least the first 24-48 hrs."

Reviewer 2, comment 13: If *Pseudoxylaria* plays a role in suppressing other fungi, what remains enigmatic is that it has such low abundance when the colony is alive. It only starts to emerge when the termites are gone. Please discuss this.

Response: This does not seem to be true as line 314 states: 'However, qPCR estimates, which are a better estimator of abundance, indicate the presence of $6.0x10^5$ copies (95% confidence intervals; Supplementary Table S6)'. This indicates that, although *Pseudoxylaria* is not visible in a fresh comb, it is still present in appreciable numbers. However, our working hypothesis to explain this discrepancy is that *Pseudoxylaria*, with its rapid growth, is a far more efficient inhibitor of non-specific fungi than *Termitomyces*. In the early stages of a comb formation, *Pseudoxylaria* can inhibit a wide range of fungi but is prevented from taking over the comb by bacterial mutualists as well as by termites. As the comb matures, this function is largely taken over by the growing colonies of *Termitomyces* which also possess some antifungal capabilities. However, *Pseudoxylaria* is never removed and remains within the comb. Any disturbance of this equilibrium, like the removal of termites, makes the conditions favorable for *Pseudoxylaria* which can then start proliferating. The qPCR data that we obtained offers some corroborating evidence towards this model. The lower abundance of *Pseudoxylaria* in comparison to *Termitomyces* can be due to its suppression by the bacterial mutualists. The same fresh comb data also indicates that *Termitomyces* numbers are at its highest. However, this is quickly reversed as termites are removed from the comb. This also indicates the role of termites in keeping *Pseudoxylaria* from proliferating. Thus, our model predicts a threshold of *Pseudoxylaria* growth which is tolerated by the termites where the fungus is forced to either grow subliminally in the comb as further proliferation would incur weeding behavior from the termites. However, this subliminal growth is enough to prevent most of the non-specific fungi as *Pseudoxylaria* is a better suppressor than *Termitomyces*. Thus, *Pseudoxylaria* continues living on the comb surviving on plant materials brought in by the worker termites and only occasionally proliferating when conditions become favorable. We are trying to work out these details in a separate manuscript.

However, we have speculated on these in lines 271-274, 291-295, 312-320.

Reviewer 2, comment 14: Line 272-274: You indicate that *Termitomyces* declines after 48 hours. I think it is reasonable to assume that this is mostly a decline, relative to *Pseudoxylaria*, rather than an absolute decline. Please discuss.

Response: It is both. There is decline in *Termitomyces* OTU's from the Nanopore data, indicated in their relative decline, as well as decline in their absolute numbers as indicated by the qPCR data.

Reviewer 2, comment 15: Line 301: change "show" to "shows"

Response: We have now changed "show" to "shows" (Line 306).

Reviewer #3

Reviewer 3, comment 2 and 15: In addition, one way to test whether Pseudoxylaria suppress additional fungi to overgrow combs is finding termite colonies that are Pseudoxylaria-free and conduct the decay experiments followed by qPCR profiling. If weed fungi overgrow decaying combs in different abundances in the absence of Pseudoxylaria, this would be compelling evidence for a possible mutualistic role of this fungus. On a side note, the manuscript does not mention whether there are termite colonies free of *Pseudoxylaria*.'

Is Pseudoxylaria present in all termite nests in a population? To test the hypothesis that *Pseudoxylaria* is competing with other fungi, instead of in vitro interactions would be more interesting to evaluate the dynamics in combs were Pseudoxylaria is not present in all stages of decay? I believe this would be the treatment to test the hypothesis.

Response: We have not found a comb without *Pseudoxylaria* yet! Each and every time we have set up a piece of fresh comb for incubation, *Pseudoxylaria* has appeared without fail. This could well mean that *O. obesus* colonies are never free of *Pseudoxylaria* as Batra and Batra (1966) in their classic study found *Pseudoxylaria* (they called it *Xylaria*) in many *Odontotermes* combs including *O. obesus*. This is of particular significance as that work was done with mounds that are not far from where we are located. Their paper also hints at an even earlier study by Bakshi (1951) which also reported *Pseudoxylaria*-like fungal growth from *O. obesus* combs. Indirectly, this can be evidence for our model which suggests that *Pseudoxylaria* is an important part of this symbiosis.

Reviewer 3, comment 3: L. 14: Why should one consider every fungus entering combs a pathogen? Perhaps, the terminology is the issue: a fungus can be a threat to combs in the sense it is a weed, but this does not

necessarily mean it is a pathogen that causes disease in combs. I agree that some fungi can be a pathogenic (or even a parasite) to combs, but label all as pathogenic seems not representative of what they really are. This discussion is important, as the whole manuscript assumes a "pathogenic load" (L. 16, 89-90 and many others). If one needs to label what these fungi are doing in combs, I would say the results of the present study support that they act as opportunists. This would be a more reasonable lifestyle for these fungi to consider.

Response: We agree with the reviewer as we also struggled to find a term for the fungi other than *Pseudoxylaria* and *Termitomyces*. We have used the term 'weedy fungi' for *Pseudoxylaria* following previous publications in the field. However, we cannot label every other fungus as 'opportunists' as we still do not know what functions, if any, that they have in this symbiosis. Therefore, we have used the term 'non-specific fungi' interchangeably with 'contaminant fungi'.

Reviewer 3, comment 4: L. 23: A hypothesis (Figure 5) derived from in vitro plate experiments seems fragile as we do not have evidence if the interactions (and gene expression) in vitro really mirror what happen in combs. Please, discuss how representative are these experiments for fresh combs.

Response: First, our in vitro plate experiments were done to confirm some of the patterns observed from real combs and not the other way round. Second, we feel that we are still some distance away from meta-transcriptomic studies as we still need to identify what we are looking for. Third, there is the added complexity of assigning an RNA transcript to a particular organism from this extremely complex ecosystem. Therefore, we have tried to break down this complexity into manageable bits of confirming the role of microbes with in vitro assays. This, we believe, is the logical way to proceed with environmental samples. We strongly feel that the 'proof' of the level of representation can only be obtained by developing an artificial comb which the termites 'accept'. Then one can inoculate specific microbes, singly or in combinations, to assess their roles in this symbiosis. Unfortunately, we have not seen any reports of this and our own efforts to formulate such an artificial comb has not succeeded yet.

However, we had alluded to this caveat earlier in lines 289-291.

Reviewer 3, comment 5: L. 26: I believe there is a distinction here regarding concepts: (i) monoculture: this is related to fungi used for food (ii) and microbe-free combs: this is related to other organisms associated with the

comb but not the cultivar. Fungus-growing insects can grow monocultures, even so in the presence of not microbe-free comb. I would consider carefully choosing between these concepts and be consist throughout the manuscript.

Response: We agree. We have now used 'disease-free' throughout the manuscript.

Reviewer 3, comment 6: L. 34 and 39: replace to "basidiomycete".

Response: We have replaced "Basidiomycota" with "basidiomycete" (Line 35 and 41).

Reviewer 3, comment 7: L. 41: I believe it is "utilizes".

Response: We have now corrected it (Line 43).

Reviewer 3, comment 8: L. 57: change to "saprotrophic" as saprophytic are more related to plants.

Response: We have now changed "saprophytic" to "saprotrophic" (Line 58).

Reviewer 3, comment 9: L. 118-119: The 0h termite-free combs also have 111 fungal genera (Table 1). It seems quite a lot just to say they are not growing in fresh combs. How indicative is it to consider these fungi are not actively growing in fresh combs? This is also a major issue that is not clear in the manuscript.

Response: We were also surprised by this number as previous reports from other termites indicate a near monopoly of *Termitomyces* from fresh combs. However, when one looks at the number of OTU's of these fungi it is clear that they are present in extremely low numbers (31-42 different fungal genera). The percent proportion of these non-specific fungi is 10% at 0 hrs which increases to 99% by the end of incubation. This perhaps indicates that they are incidental contaminants and/or have been suppressed in the active comb as no other fungi is visible on the surface of a fresh comb.

Reviewer 3, comment 10: L. 124: I really miss figures with the termite workers, general nest structure and fungus combs to illustrate the study system. Perhaps, bring some of the beautiful photos in the supplementary material to the main manuscript.

Response: We have now included the figures of the termite colony as a major figure in Fig. 1.

Reviewer 3, comment 11: L. 224: "fungal genera" instead of "fungal strains" according to Table 1?

Response: We have changed "fungal strains" to "fungal genera" in the revision (Line 226).

Reviewer 3, comment 12: L. 241: I believe also *Termitomyces* and bacteria as shown in this study, right?

Response: We agree. We have updated the statement in line 244-246 to:

"Consequently, these results indicate the efficiency of termites suppressing the growth of non-cultivar fungi, together with the efficiency of *Termitomyces* and bacterial strains for the successful proliferation of termite colonies" (Line 244-246).

Reviewer 3, comment 13: L. 472-473: Why make this selection? Would it make more sense to have used all the other fungal contaminants against the bacteria too?

Response: Our goal here was to find whether there is at least a one-to-one relationship between a non-specific fungus and a bacterial mutualist. This would illuminate our contention that this symbiosis is well equipped to deal with any fungal invader. We have been largely successful in showing that there is indeed such a relationship. However, this does not assume that these bacteria cannot prevent other fungi and we have never claimed that. If they can, then it provides further evidence for our model in showing that there are several microbes which can take up the role of preventing invading fungi.

Reviewer 3, comment 14: Figure 1: There are no numbers under the pie charts. Also, are the genera highlighted in blue or in bold?

Response: We have now mentioned the relative abundances of *Termitomyces* and other fungi below the pie chart. The highlighted genera are in bold. We have corrected this information in the legend of figure 2 (figure 1 is updated to figure 2).

Reviewer 3, comment 16: Figure 3: ITS is not a gene.

Response: We have corrected it in line 371 and in legend of figure 4 (figure 3 is now figure 4).

Reviewer 3, comment 17: Figure 5: Name "bacterial mutualists" means that bacteria are benefiting from the association. Is this true for all bacteria examined in the present study? Do we know the reciprocal benefits of bacteria and Termitomyces for all bacteria found in combs. I believe it is better to specify which bacteria this manuscript is building the hypothesis.

Response: We still do not know how the bacteria are benefitting from this association. The list of bacteria used comes from our previous study (Agarwal et al, 2021) which found these bacteria. These are mentioned in lines 487-490. We have also mentioned the names of bacterial genera in the figure 6.

Reviewer 3, comment 18: Table 1: The manuscript will read better if the decay stage 0h, which is really not a decay treatment yet, could be renamed to "fresh comb".

Response: We agree. We have now changed "0 hrs old comb" to "fresh comb (0 hrs)" in the entire manuscript including figures.

Reviewer 3, comment 19: Table 2: This table could go in the supplementary since Figure 4 summarizes the results already.

Response: We have now moved table 2 to supplementary as table S8.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I don't think the author has gained rigor conclusion "Partnership with both fungi and bacteria can protect Odontotermes obesus fungus gardens against fungal invaders". The inhibition activities of the tested bacterial mutualists、Termitomyces and Pseudoxylaria against the most of fungal contaminants were weak. The author did not find any innovative conclusions.So I recommend to you that this manuscript can not be accepted.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments satisfactorily.

Reviewer #4 (Remarks to the Author):

Reviewer comments
This manuscript explores the diverse microbial community of both bacteria and fungi that fungus-farming termite combs harbors. The paper suggests that not only the bacterial and fungal symbiont, Termitomyces, but also Pseudoxylaria are part of the defensive barrier that protects and keeps the colony free from unwanted infections and contaminants. The antifungal activity is supported by growth plate assays of both bacterial symbionts together with the fungal crop Termitomyces and the opportunistic fungi Pseudoxylaria. The results show that both bacteria and fungi have varying and overlapping degrees of antifungal activity and the indicates that's it is the combined effect of these bacterial symbionts and the fungal crop that suppresses the growth of potential antagonistic fungi within the fungal combs.

Overall, I found the paper interesting and well written. The colony sample size, as the other reviewer mentioned, is quite low, but overall, the science seems robust. I was asked to provide feedback on how suitably Reviewer #3's comments have been addressed, and whether the manuscript is suitable for publication, this is what I will focus on. I have a few additional comments of my own (see below), that I hope will help to further improve the manuscript.

My biggest concern is with the suggestion that your in vitro study can be compared to what happens in vivo. It is a common problem in both plant and mycology studies that what we observe in the lab cannot be transferred to what happens in the field without further testing. The hypothesis that Pseudoxylaria aids in keeping the symbiosis free from diseases needs to be reevaluated.

Specific comments, with recommendations for addressing each comment.
If Reviewer #3's comments have been addressed properly I will simply write "ok.". If I have additional comments or suggestions these will be noted. I have some general changes to the text throughout the manuscript. These can be ignored if the authors do not agree.

I found that many of the comments from reviewer #3 have been adequately addressed. I would suggest some more revisions to address the remaining comments before the manuscript is ready for publications.

Reviewer 3, comment 1:
I agree with all reviewers that the study could have benefited from additional colony sampling. However, as someone who also goes into the field and collect from colonies of this subfamily of termite, I can appreciate the authors desire to not further stress and potentially kill additional termite mounds. As the paper stands you need to further highlight this sampling caveat throughout the paper. I would do so in both method and results. You already have your arguments in your rebuttal letter, so just add a sentence addressing why you only sampled from two colonies.
I think the authors have addressed this concern accurately in the discussion with the additional lines provided at the end of the paper.

Reviewer 3, comment 2 and 15:
Pseudoxylaria is a known specialist of fungus-farming termites. A specialist does not mean that is an important part of a symbiosis ifself. Many symbioses have specialised pathogens. Pseudoxylaria is almost always present in the comb but not growing in healthy colonies. I agree with the authors here.

Reviewer 3, comment 3: L. 14
I see the issue with the terminology. Neither weedy nor pathogenic fungi are an accurate term for the potential fungal contaminants that the termites bring home from their foraging activities as you don't know how they would affect the colony and comb. You are using "weedy fungi" on line 57,59,62 (and many more) when referring to the environmental fungal contaminants, but in your rebuttal letter you say that you will be using the term "weedy fungi" for Pseudoxylaria. I think the term you mention in your rebuttal letter "non-specific fungi" when referring to fungi other than Termitomyces and Pseudoxylaria would be sufficient, and this should be incorporated into the text. Then you have three clear descriptives: Termitomyces = fungal cultivar, Pseudoxylaria = weedy fungi, environmental fungi = non-specific fungi. But these three descriptives needs to be made clear early in the text to help the reader.
For example: L83: do you mean only the non-specific fungi or also Pseudoxylaria. This is not clear to me.
I would change line 57 and add a line explaining the use of non-specific fungi. My suggestion:
"… increases the threat of introducing potential fungal contaminants from the environment. These fungi will hereafter be referred to as "non-specific fungi" throughout the text."

Reviewer 3, comment 4: L. 23:
Your hypothesis derived from in vitro plates is not supported by your results.
Pseudoxylaria is only present in healthy mound in such small quantities that I fail to see how it can protect the colonies when they are healthy. The genus Xylaria is known for its "sit and wait" strategy. Not only in fungus combs but also on other organisms e.g. trees. Once a branch falls off, Xylaria will start to grow. But only when the "tree"-part is dead or dying. Species within this genus are major producers of antimicrobial specialised metabolites, and antifungal activity is something that have

been shown in numerous studies for these types of fungi. Therefore, the presence of antimicrobial activity in Pseudoxylaria based on the in vitro plate experiment assays does not provide compelling enough evidence to suggest a mutualistic role of Pseudoxylaria within fungus-farming termites.
Some refs:
https://doi.org/10.1038/s41396-023-01374-4
https://doi.org/10.1016/j.funeco.2011.05.003
https://doi.org/10.1039/D1NP00022E

Your growth assay proves that Pseudoxylaria have antifungal activities when it is in its growing state = in decaying colonies. But not the antifungal potential of Pseudoxylaria in healthy colonies, when Pseudoxylaria is not growing. Therefore, your results indicates that the antifungal potential of Pseudoxylaria against environmental fungi (which are also present in the comb) aids Pseudoxylaria in being the fastest to overgrow the nutrient-rich resource that is the fungus combs once the colony is in decay. This is also interesting. Pseudoxylaria has previously been tested in vitro and have shown antimicrobial potential against multiple bacterial and fungal strains. However, to my knowledge this study is the first to test both Termitomyces and Pseudoxylaria antifungal potential against ecological relevant fungal strains isolated directly from fungus combs. This is the interesting and novel part of your study and this needs to be highlighted more.
For example: L196-197: "… these results indicate that Termitomyces possess the capability of preventing many ecological relevant fungal contaminants to a variable degree".

L221-222: You cannot state that Pseudoxylaria takes part in preventing the spread of other fungi within the comb in collaboration with the termites and their symbionts. As you only tested Pseudoxylaria in vitro and when it was growing ◊ mimicking the decaying state and not the healthy colony state. This needs to be rewritten.

L254-256: No, you cannot state that there is a synergistic effect of Termitomyces and Pseudoxylaria based on your results.
Pseudoxylaria most likely show more antifungal effects due to its "sit-and-wait" strategy. Once the comb resource is available (decaying colony) it quickly takes over, outcompeting all the other fungi present in the comb.

In conclusion, you need to reevaluate your hypothesis that Pseudoxylaria is a potential mutualist of the symbiosis as your results do not support this.

Reviewer 3, comment 5: L. 26:
Ok

Reviewer 3, comment 6: L. 34 and 39:
Ok.

Reviewer 3, comment 7: L. 41:
Ok.

Reviewer 3, comment 8: L. 57:
Ok

Reviewer 3, comment 9: L. 118-119:
This is not too surprising as previous studies have also identified many different fungal spores within fungus comb, but not actively growing colonies.
Refs that can be added to your own observation:
Moriya et al. 2015; http://dx.doi.org/10.1264/jsme2.20.243
Otani et al. 2019: https://doi.org/10.1038/s41598-019-45364-z
Thomas 1987: https://doi.org/10.1016/0038-0717(87)90019-8

Reviewer 3, comment 10: L. 124:
Ok.

Reviewer 3, comment 11: L. 224:
Ok.

Reviewer 3, comment 12: L. 241:
Ok. I would potentially change the sentence to:
“Consequently, these results indicate the efficiency of termites suppressing the growth of non-cultivar fungi, together with the efficiency of Termitomyces and bacterial strains. These combined actions of these microbial symbionts ensure successful proliferation of healthy termite colonies” (Line 244-246).

Reviewer 3, comment 13: L. 472-473:
I understand your reasons for selecting these fungal strains. However, this overlap in antifungal activity should be mentioned in the discussion. For example, L330.

Reviewer 3, comment 14: Figure 1 (now 2):
Ok.

Reviewer 3, comment 16: Figure 3 (now 4):
Ok.

Reviewer 3, comment 17: Figure 5 (now 6):
Ok.

Reviewer 3, comment 18: Table 1:
Ok.

Reviewer 3, comment 19: Table 2:
Ok.

In addition to the comment from reviewer #3 I have a very few of my own regarding the figures:

Comment 20: Figure 1,3,5 and 6: all are blurry when you zoom in. Is it possible to increase the quality of the figures?

Comment 21: Figure 2: The list of fungal genera does not align perfectly to the heatmap, which makes it harder to interpret. I suggest that you fix that.

Comment 22, the competition assays setup: I would be careful with saying that in vitro plates only grown for 36 hours of pre-incubation mimics that of real colony with dominating Termitomyces. For future studies, maybe a week or two, would have been a better representation of what happens within an established colony where there is a huge biomass of Termitomyces present within the fungus combs.

Possible text changes:
L60: “Many of these have the potential to outcompete the fungal cultivar, Termitomyces …”
L71: you have a space between the reference number 29 and insects.
L77: “…Pseudoxylaria, the most prevalent non-Termitomyces fungal growth observed within decaying termite fungal gardens.
Note: Pseudoxylaria and other fungi spores have been identified in these fungal gardens but only as spores and not as growing cultures. It is only when the termites are removed, or the colony is dying that we see this appearance of Pseudoxylaria in the combs.

Response to the reviewers

We thank the reviewers for their assessment of our work. Below, we present a detailed response to the reviewers' comments:

Reviewer #1

I don't think the author has gained rigor conclusion "Partnership with both fungi and bacteria can protect *Odontotermes obesus* fungus gardens against fungal invaders". The inhibition activities of the tested bacterial mutualists, *Termitomyces* and *Pseudoxylaria* against the most of fungal contaminants were weak. The author did not find any innovative conclusions. So I recommend to you that this manuscript can not be accepted.

Response: We are ready to make improvements if the reviewer has any specific suggestions for the manuscript.

Reviewer #4

The comments and responses are in the following order: Firstly we have addressed reviewer 3 comments which were pointed out again by Reviewer 4, then we have addressed Reviewer 4 own comments and lastly minor text changes suggested by Reviewer 4.

Reviewer 3 (4), general comment: My biggest concern is with the suggestion that your in vitro study can be compared to what happens *in vivo*. It is a common problem in both plant and mycology studies that what we observe in the lab cannot be transferred to what happens in the field without further testing. The hypothesis that *Pseudoxylaria* aids in keeping the symbiosis free from diseases needs to be reevaluated.

Response: We generally agree with this comment and have previously indicated in our manuscript of this caveat (Lines 269-271, 296-298, 304-306, 343-347). However, our evidence of the activity of *Pseudoxylaria*, especially against *Termitomyces*, follows *in vivo* trials and not the other way

around. We show that when termites are removed from a fresh comb, *Pseudoxylaria* rapidly displaces *Termitomyces*. Our *in vitro* experiments with these two fungi confirm what we observe from these decaying combs. This is also seen in the activity of the many non-specific fungi vis-à-vis *Pseudoxylaria*.

The fungus combs are host to extremely complex communities of microbes. This complexity makes the application of Koch's postulates, the gold standard of establishing the role of a microbe to a particular disease, very difficult. However, most natural microbial communities are similarly complex with most microbes beyond the scope of culturing. But these difficulties do not hinder the formation of cogent scientific observations provided there are multiple lines of evidence confirming an observed phenomenon. Therefore, we have been extremely conservative in our conclusions and have presented it as a hypothesis to be evaluated.

Reviewer 3 (4), comment 1: I agree with all reviewers that the study could have benefited from additional colony sampling. However, as someone who also goes into the field and collect from colonies of this subfamily of termite, I can appreciate the authors desire to not further stress and potentially kill additional termite mounds. As the paper stands you need to further highlight this sampling caveat throughout the paper. I would do so in both method and results. You already have your arguments in your rebuttal letter, so just add a sentence addressing why you only sampled from two colonies. I think the authors have addressed this concern accurately in the discussion with the additional lines provided at the end of the paper.

Response: We have now added the lines in methods and results section to highlight the use of two mounds in Lines 126-129, 371-373, 438-443.

Reviewer 3 (4), comment 2 and 15:

Pseudoxylaria is a known specialist of fungus-farming termites. A specialist does not mean that is an important part of a symbiosis ifself. Many symbioses have specialised pathogens.

Pseudoxylaria is almost always present in the comb but not growing in healthy colonies. I agree with the authors here.

Response: We thank reviewer for this comment.

Reviewer 3 (4), comment 3: L. 14

I see the issue with the terminology. Neither weedy nor pathogenic fungi are an accurate term for the potential fungal contaminants that the termites bring home from their foraging activities as you don't know how they would affect the colony and comb. You are using "weedy fungi" on line 57,59,62 (and many more) when referring to the environmental fungal contaminants, but in your rebuttal letter you say that you will be using the term "weedy fungi" for *Pseudoxylaria*. I think the term you mention in your rebuttal letter "non-specific fungi" when referring to fungi other than *Termitomyces* and *Pseudoxylaria* would be sufficient, and this should be incorporated into the text. Then you have three clear descriptives: *Termitomyces* = fungal cultivar, *Pseudoxylaria* = weedy fungi, environmental fungi = non-specific fungi. But these three descriptives needs to be made clear early in the text to help the reader. For example: L83: do you mean only the non-specific fungi or also *Pseudoxylaria*. This is not clear to me. I would change line 57 and add a line explaining the use of non-specific fungi. My suggestion:"… increases the threat of introducing potential fungal contaminants from the environment. These fungi will hereafter be referred to as "non-specific fungi" throughout the text."

Response: We agree with the reviewer. We have now changed the term 'weedy fungi' to 'non-specific fungi' for all except *Termitomyces* and *Pseudoxylaria*. These fungi will hereafter be referred to as "non-specific fungi" throughout the text and figures. We have also changed the line 56 to:

Additionally, the continuous and unidirectional flow of dead and decaying plant materials into these mounds increases the threat of introducing potential non-specific fungi from the environment. (line 56- 58)

Reviewer 3 (4), comment 4: L. 23, Part 1:

Your hypothesis derived from *in vitro* plates is not supported by your results. *Pseudoxylaria* is only present in healthy mound in such small quantities that I fail to see how it can protect the colonies when they are healthy. The genus *Xylaria* is known for its "sit and wait" strategy. Not only in fungus combs but also on other organisms e.g. trees. Once a branch falls off, *Xylaria* will start to grow. But only when the "tree"-part is dead or dying. Species within this genus are major producers of antimicrobial specialised metabolites, and antifungal activity is something that have been shown in numerous studies for these types of fungi. Therefore, the presence of antimicrobial activity in *Pseudoxylaria* based on the *in vitro* plate experiment assays does not provide compelling enough evidence to suggest a mutualistic role of *Pseudoxylaria* within fungus-farming termites.

Response: We agree that the high-throughput data, from the Nanopore platform, indicates *Pseudoxylaria* OTU's to be scarce in a fresh comb (Line 141-144). This is generally in-sync with other studies (lines 237-244). However, our qPCR analysis, using the same DNA samples used for Nanopore sequencing, updates that abundance to substantially higher levels (Supplementary table S6). These two types of data, although differing in their magnitude, shows a simple correlation (fig 3). This indicates that, as expected, qPCR is a far more sensitive and efficient estimator of abundance for individual microbes. Therefore, our efforts towards methodological improvement to detect *Pseudoxylaria* have now paid off as it has given a clearer picture of its abundance. Second, the 'sit-and-wait' strategy of *Pseudoxylaria* raises more questions than it answers. This is especially relevant with our data (both *in-vitro* as well as *in-vivo*) showing *Pseudoxylaria* can easily take over *Termitomyces*. Why 'sit-and-wait' if it can easily take over *Termitomyces*? Which microbe or what forces keep it under check in a healthy comb? How does *Pseudoxylaria* remain viable for the duration of its 'sit-and-wait' phase and not displaced by other microbes? A more parsimonious explanation than 'sit-and-wait' is that *Pseudoxylaria* keeps on flourishing but is prevented from taking over a comb by the termite workers and bacterial mutualists. For, only upon the removal of termites do the proliferation of *Pseudoxylaria* begins. If this is true, then the growing *Pseudoxylaria* can keep several other non-specific fungi at bay. This remains the gist of our argument and the basis of our hypothesis.

We had answered a similar question from Reviewer#2 (comment 13) in the first revision. Here we are reproducing the same.

Reviewer 2, comment 13*: If Pseudoxylaria plays a role in suppressing other fungi, what remains enigmatic is that it has such low abundance when the colony is alive. It only starts to emerge when the termites are gone. Please discuss this.*

Response: *This does not seem to be true as line 318 (now line 321-322) states: 'However, qPCR estimates, which are a better estimator of abundance, indicate the presence of* $6.0x10^5$ *copies (95% confidence intervals; Supplementary Table S6)'. This indicates that, although Pseudoxylaria is not visible in a fresh comb, it is still present in appreciable numbers. However, our working hypothesis to explain this discrepancy is that Pseudoxylaria, with its rapid growth, is a far more efficient inhibitor of non-specific fungi than Termitomyces. In the early stages of a comb formation, Pseudoxylaria can inhibit a wide range of fungi but is prevented from taking over the comb by bacterial mutualists as well as by termites. As the comb matures, this function is largely taken over by the growing colonies of Termitomyces which also possess some antifungal capabilities. However, Pseudoxylaria is never removed and remains within the comb. Any disturbance of this equilibrium, like the removal of termites, makes the conditions favorable for Pseudoxylaria which can then start proliferating. The qPCR data that we obtained offers some corroborating evidence towards this model. The lower abundance of Pseudoxylaria in comparison to Termitomyces can be due to its suppression by the termites with the help of bacterial mutualists. The same fresh comb data also indicates that Termitomyces numbers are at its highest. However, this is quickly reversed as termites are removed from the comb. This also indicates the role of termites in keeping Pseudoxylaria from proliferating. Thus, our model predicts a threshold of Pseudoxylaria growth which is tolerated by the termites where the fungus is forced to either grow subliminally in the comb as further proliferation would incur weeding behavior from the termites. However, this subliminal growth is enough to prevent most of the non-specific fungi as Pseudoxylaria is a better suppressor than Termitomyces (Fig. 4). Thus, Pseudoxylaria continues living on the comb surviving on plant materials brought in by the worker termites and only occasionally proliferating when conditions become favorable. We are trying to work out these details in a separate manuscript.*

However, we have speculated on these in lines 279-281, 303-306, 322-329.

Reviewer 3 (4), comment 4: L. 23, Part 2:

Some refs:

https://doi.org/10.1038/s41396-023-01374-4
https://doi.org/10.1016/j.funeco.2011.05.003
https://doi.org/10.1039/D1NP00022E

Your growth assay proves that *Pseudoxylaria* have antifungal activities when it is in its growing state = in decaying colonies. But not the antifungal potential of *Pseudoxylaria* in healthy colonies, when *Pseudoxylaria* is not growing. Therefore, your results indicates that the antifungal potential of *Pseudoxylaria* against environmental fungi (which are also present in the comb) aids *Pseudoxylaria* in being the fastest to overgrow the nutrient-rich resource that is the fungus combs once the colony is in decay. This is also interesting. *Pseudoxylaria* has previously been tested *in vitro* and have shown antimicrobial potential against multiple bacterial and fungal strains. However, to my knowledge this study is the first to test both *Termitomyces* and *Pseudoxylaria* antifungal potential against ecological relevant fungal strains isolated directly from fungus combs. This is the interesting and novel part of your study and this needs to be highlighted more.

For example: L196-197: "… these results indicate that *Termitomyces* possess the capability of preventing many ecological relevant fungal contaminants to a variable degree".

Response: We have cited these references in the manuscript now (line 71, 310 and 306). We also thank the reviewer for this comment. We have added the following lines emphasizing this fact:

"However, these results indicate that *Termitomyces* possess the capability of preventing many ecologically relevant non-specific fungi to a variable degree." (Lines 203-204).

L221-222: You cannot state that *Pseudoxylaria* takes part in preventing the spread of other fungi within the comb in collaboration with the termites and their symbionts. As you only tested *Pseudoxylaria in vitro* and when it was growing mimicking the decaying state and not the healthy colony state. This needs to be rewritten.

Response: Our current work is based on what happens when termites are removed from a functioning and healthy comb. Disturbing this equilibrium was crucial as we wanted to know what maintains this healthy state. This is not unlike a mutant genotype illuminating the functions of the wildtype and/or trying to understand a disease phenotype to know what maintains a healthy state. On comparison of the data obtained from decaying stages with the fresh comb did we formulate our hypothesis. Given the various data obtained it is our conjecture that *Pseudoxylaria* could be a partner in this symbiosis (lines 296-337) and we presented this as a hypothesis/verbal model which is open for other groups to invalidate with better data. We have stated that this is a controversial idea (line 292-293) and reiterated that this requires more data to be validated (lines 345-347). If one cannot present a hypothesis based on the results obtained then it is difficult to run a scientific program. However, we have modified line 227 to:

"These *in vitro* interaction assays suggest that termites possess multifaceted capability of preventing the growth of non-specific fungi through *Termitomyces*, *Pseudoxylaria* and bacterial mutualists."

L254-256: No, you cannot state that there is a synergistic effect of *Termitomyces* and *Pseudoxylaria* based on your results.

Pseudoxylaria most likely show more antifungal effects due to its "sit-and-wait" strategy. Once the comb resource is available (decaying colony) it quickly takes over, outcompeting all the other fungi present in the comb.

In conclusion, you need to reevaluate your hypothesis that *Pseudoxylaria* is a potential mutualist of the symbiosis as your results do not support this.

Response: First, the synergistic effect of *Termitomyces* and *Pseudoxylaria* was proposed after we confirmed it with a statistical test (Fig. S7). Second, we have stated above our views on the 'sit-and-wait' strategy and how we find it inadequate in explaining the behaviour of *Pseudoxylaria*. Third, our data shows that *Pseudoxylaria* itself is displaced by other fungi as the process of decay continues (Main figure 3, *Pseudoxylaria* OTU experience a reduction of 26% to 12%). This indicates that either some microbe is capable of preventing it and/or is better at utilizing the available resources than *Pseudoxylaria*. This indicates that the comb might have microbes which can out-compete *Pseudoxylaria* and it runs the risk of being eliminated if its only strategy is 'sit-and-wait'.

However, we have modified lines 343-345 to:

"This model, however, awaits empirical validation as several aspects of *Pseudoxylaria* functioning remains unclear like its role during the formation of a comb as it necessitates the demonstration of the anti-fungal effects of *Pseudoxylaria*[48] even in these early stages."

Reviewer 3 (4), comment 9: L. 118-119:

This is not too surprising as previous studies have also identified many different fungal spores within fungus comb, but not actively growing colonies. Refs that can be added to your own observation:

Moriya et al. 2015; http://dx.doi.org/10.1264/jsme2.20.243

Otani et al. 2019: https://doi.org/10.1038/s41598-019-45364-z

Thomas 1987: https://doi.org/10.1016/0038-0717(87)90019-8

Repsonse: We have now added these references in lines 83 and 120.

Reviewer 3 (4), comment 12: L. 241:

Ok. I would potentially change the sentence to:

“Consequently, these results indicate the efficiency of termites suppressing the growth of non-cultivar fungi, together with the efficiency of Termitomyces and bacterial strains. These combined actions of these microbial symbionts ensure successful proliferation of healthy termite colonies” (Line 244-246).

Response: We have now changed the line according to reviewer suggestion (Line 252-254).

Reviewer 3 (4), comment 13: L. 472-473:

I understand your reasons for selecting these fungal strains. However, this overlap in antifungal activity should be mentioned in the discussion. For example, L330.

Response: We have the following line in the discussion (266-269):

“Moreover, the eight bacterial mutualists previously identified[33] also show similar growth inhibitory capabilities. Thus, our results point to the presence of microbes, both bacterial and fungal, with fungicidal capabilities present in this symbiosis.”

In addition to the comment from reviewer #3 I have a very few of my own regarding the figures:

Reviewer 4, Comment 20: Figure 1,3,5 and 6: all are blurry when you zoom in. Is it possible to increase the quality of the figures?

Response: We have now increased the resolution of each figure. However, we do not have higher resolution pictures from the field (Fig. 1) as these were taken without a macro lens.

Reviewer 4, Comment 21: Figure 2: The list of fungal genera does not align perfectly to the heatmap, which makes it harder to interpret. I suggest that you fix that.

Response: We have now fixed the problem of alignment in figure 2.

Reviewer 4, Comment 22, the competition assays setup: I would be careful with saying that *in vitro* plates only grown for 36 hours of pre-incubation mimics that of real colony with dominating *Termitomyces*. For future studies, maybe a week or two, would have been a better representation of what happens within an established colony where there is a huge biomass of *Termitomyces* present within the fungus combs.

Response: We agree that 36 hr timeframe may not precisely mimic the dominant *Termitomyces* in a real colony. However, growing *Termitomyces* for a week or two before introducing the competing fungus may not yield accurate results as any inhibition seen can be attributed to the depletion of nutrients from the media or *Termitomyces* dying than to the antifungal activity of *Termitomyces*. However, we would like to emphasize that even after just 36 hours of growth we could still observe inhibition by *Termitomyces*.

Possible text changes:

L60: "Many of these have the potential to outcompete the fungal cultivar, Termitomyces …"

Response: We have now changed the line 60 to:

"Many of these have the potential to outcompete the fungal cultivar *Termitomyces* and can result in a severe reduction of available nutrients for the termites."

L71: you have a space between the reference number 29 and insects.

Response: We have removed the space between reference and insects in line 72.

L77: "…*Pseudoxylaria*, the most prevalent non-*Termitomyces* fungal growth observed within decaying termite fungal gardens.

Note: *Pseudoxylaria* and other fungi spores have been identified in these fungal gardens but only as spores and not as growing cultures. It is only when the termites are removed, or the colony is dying that we see this appearance of *Pseudoxylaria* in the combs.

Response: We have changed the text in line 78 to:

"Accordingly, some *in vitro* studies have found many resident bacteria acting as secondary mutualists capable of preventing *Pseudoxylaria*[34-36], the most prevalent non-*Termitomyces* fungal growth observed within decaying termite fungal gardens."

However, we would like to state that we could not find any studies which clearly distinguished the presence of spores from growing cultures for any particular fungi. Spores can be present in fungus combs as contamination. But this also presents another ambiguity as spores must be prevented from germinating. This essentially means that germination is suppressed by either the termites or by the unfavorable conditions within a comb. The latter seems implausible as the combs have ideal growing conditions for many fungi. The former conjecture is also non-parsimonious as termites must selectively prevent the germination of all non-*Termitomyces* spores and only allow the asexual spores of *Termitomyces* to germinate.

Reviewers' comments:

Reviewer #4 (Remarks to the Author):

Reviewer 4

Overall
The inhibition activities and identification of Pseudoxylaria (and the others) markers are not strong enough to support your hypothesis/discussion. As the manuscript stands I cannot recommend that it should be accepted.

Comment 1:
As per my previous comment it is the caveat of only sampling from two colonies that you needed to highlight not the number of samples from these two colonies. You are stating how much fungus comb and termites you have. Not that these samples only originate from 2 colonies.

Comment 2:
Ok.

Comment 3:
Ok.

Comment 4:
As per my previous comment, this “sit and wait” strategy is a known phenomenon of Xylaria and your results does not provide evidence to the contrary.
One limitation of using changes in copy numbers of microorganisms determined by quantitative polymerase chain reaction (qPCR) analysis is that it provides indirect information about microbial abundance. While qPCR can accurately measure the relative abundance of specific DNA sequences (such as genes or genomic regions) in a sample, it does not directly quantify the number of viable microorganisms present. Instead, it measures the abundance of specific genetic markers associated with target microorganisms. Therefore, while you might be identifying the presence of Pseudoxylaria markers you are not actually measuring active and growing Pseudoxylaria.

It is important to know that qPCR analysis cannot provide a comprehensive understanding of microbial dynamics, as it does not differentiate between viable and non-viable microorganisms or account for factors such as metabolic activity or growth rates. Additionally, qPCR may be influenced by factors such as DNA extraction efficiency, PCR inhibitors, and variations in target gene copy numbers among different microbial species or strains.
While qPCR is a very valuable tool, its results should be interpreted cautiously and in conjunction with other microbial community analysis techniques to provide a more complete understanding of microbial ecology.

Your qPCR (of genomic material) and the in vitro inhibition assays are showing two very different growth stages of Pseudoxylaria.

What your growth assay is showing is Pseudoxylaria's production of antimicrobial compounds with the ability to limit the growth of non-Termitomyces fungi to ensure Pseudoxylarias proliferation on the new available substrate. Which is the known strategy of Xylaria fungi.
To sum up: The presence of antimicrobial activity in Pseudoxylaria based on the qPCR and the growth assays does not provide compelling enough evidence to suggest a mutualistic role of Pseudoxylaria within fungus-farming termites and that this hypothesis that Pseudoxylaria aids in keeping the symbiosis free from diseases needs to be reevaluated before the manuscript can be submitted.

As per my previous comment, your results indicates that the antifungal potential of Pseudoxylaria against environmental fungi aids Pseudoxylaria in being the fastest to overgrow the newly available nutrient-rich resource once the colony is in decay. From what I can find, this study is the first to test Pseudoxylaria antifungal potential against ecological relevant fungal strains isolated directly from fungus combs. This is the novelty of your study.

Comment 4 – part 2:
Line 296-298: "…if the anti-fungal capabilities of Pseudoxylaria and Termitomyces, as seen 296 in culture assays (Fig. 4), are a reliable indicator of their capabilities within a fungus comb, then it 297 is clear that Termitomyces is a less efficient inhibitor of non-specific fungi than Pseudoxylaria."
But is it not a reliable indicator of what happens inside healthy colonies. The culture assays show a growing and active Termitomyces (healthy combs) and Pseudoxylaria (decaying comb)
Line 292-293: Yet you spend line 293 – 327 defending this controversial hypothesis that your results do not support. Numerous studies have shown that Pseudoxylaria and Termitomyces thrive in the same growth conditions. This yet again supports the general strategy of Xylaira fungi as opportunistic fungi that take over substrate once it becomes available.

Comment 9:
Ok.

Comment 12:
Ok.

Comment 13:
Ok.

Comment 20:
Ok.

Comment 21:
Ok.

Comment 22:
The fact that you see inhibition of Termitomyces even after 36 hours of growth is very interesting and I would suggest you highlight/explore this further.

Comment 23:
“This essentially means that germination is suppressed by either the termites or by the unfavorable conditions within a comb. The latter seems implausible as the combs have ideal growing conditions for many fungi”.
More and more studies are highlighting the holistic approach of many symbioses to prevent the growth of antagonists. Within fungus-farming termites it is not only the symbiosis members that are responsible for ensuring the proliferation of Termitomyces and inhibition of non-Termitomyces. The termite ensures optimal growth condition for Termitomyces and the inner environment (high CO_2) of the colony itself have been shown to affect the growth and germination of unwanted fungi.
Ref: https://doi.org/10.3389/fevo.2023.1134492

Response to the reviewers

Response to the reviewers

We thank the reviewers for their assessment of our work. Below, we present a detailed response to the reviewers' comments:

Comment 1: As per my previous comment it is the caveat of only sampling from two colonies that you needed to highlight not the number of samples from these two colonies. You are stating how much fungus comb and termites you have. Not that these samples only originate from 2 colonies.

Response: We have mentioned this several times including in the discussion (lines 127,359, 361, 380, 387, and 442). These are the lines 367-373:

"Some of the experiments detailed here are relatively difficult to do because they involve sampling substantial amounts of fungus combs and consequently, only large and mature colonies can be used, which are few and far between. Therefore, these studies need to be confirmed with additional sampling from different fungus-growing termites. Thus, the role of *Pseudoxylaria* in fungus-termite system is mostly considered that of a weed and threat to termite colony unless concrete evidence emerges to the contrary."

Comment 4, part 1: As per my previous comment, this "sit and wait" strategy is a known phenomenon of *Xylaria* and your results does not provide evidence to the contrary.One limitation of using changes in copy numbers of microorganisms determined by quantitative polymerase chain reaction (qPCR) analysis is that it provides indirect information about microbial abundance. While qPCR can accurately measure the relative abundance of specific DNA sequences (such as genes or genomic regions) in a sample, it does not directly quantify the number of viable microorganisms present. Instead, it measures the abundance of specific genetic markers associated with target microorganisms. Therefore, while you might be identifying the presence of *Pseudoxylaria* markers you are not actually measuring active and growing *Pseudoxylaria*.

Response: We have never stated anywhere in the manuscript that we are assaying live and viable *Pseudoxylaria* cells from the fungus combs. That requires other molecular techniques. However, our qPCR data is consistent with what we observe happening in the combs, namely, the sudden decrease in *Termitomyces* and a concomitant increase in *Pseudoxylaria* abundance. However, if primers designed towards 'specific genetic markers' from *Pseudoxylaria* indicate relatively high numbers then there is a good chance of having relatively high

Pseudoxylaria numbers! There is very little wiggle room for us to interpret it otherwise. However, we have added the following line (Lines 324-325)

"However, whether these numbers represent actively growing *Pseudoxylaria* or not remains to be seen."

Comment 4, part 2: It is important to know that qPCR analysis cannot provide a comprehensive understanding of microbial dynamics, as it does not differentiate between viable and non-viable microorganisms or account for factors such as metabolic activity or growth rates.

Response: We fully agree. PCR and qPCR cannot adequately represent metabolic activity nor can it distinguish between dead and live cells. However, it can give an estimate of growth rates as increase in the number of cells (or growth) can produce higher qPCR values unless that growth is accompanied by a lack of increase in the cell nuclei. Therefore, the contention that qPCR cannot estimate normal tissue growth is non-parsimonious.

Comment 4, part 3: Additionally, qPCR may be influenced by factors such as DNA extraction efficiency, PCR inhibitors, and variations in target gene copy numbers among different microbial species or strains. While qPCR is a very valuable tool, its results should be interpreted cautiously and in conjunction with other microbial community analysis techniques to provide a more complete understanding of microbial ecology.

Response: Again, we agree that all these factors can influence qPCR results as we have been using qPCR since 2010. As detailed in supplementary Note 2 we have used the same DNA for qPCR with which we have ran our Nanopore platform. If there were PCR inhibitors then it would influence both reactions equally. Moreover, PCR inhibitors would reduce the numbers seen in our qPCR reactions and not increase them. Also, the target sequences for these primers were the same ITS region with which we have done our Nanopore reactions. Again, if target gene copy numbers were an issue, then it would have influenced both reactions. We cited the Zhang & Fang (2006) paper (Reference #45) which elaborates why qPCR is a better estimator of microbial abundance and will not elaborate it here.

Comment 4, part 4: We have answered this part at the end.

Response to the reviewers

Comment 5: Comment 4 – part 2: Line 296-298: "…if the anti-fungal capabilities of *Pseudoxylaria* and *Termitomyces*, as seen 296 in culture assays (Fig. 4), are a reliable indicator of their capabilities within a fungus comb, then it 297 is clear that *Termitomyces* is a less efficient inhibitor of non-specific fungi than *Pseudoxylaria*." But is it not a reliable indicator of what happens inside healthy colonies. The culture assays show a growing and active *Termitomyces* (healthy combs) and *Pseudoxylaria* (decaying comb)

Response: We do not understand this comment. However, we understand that sometimes the microbes may behave differently *in vitro* compared to *in vivo.* But in most fungus-growing insects, assumptions about the functionality of symbiotic association have been made based on the evidence gathered from *in vitro* assays. Considering the technical difficulties of conducting these assays *in vivo*, one can only replicate them *in vitro* with the reservation that the microbes may behave differently. Therefore, we have followed the same assumptions and qualified our findings with "…**if** the anti-fungal capabilities of *Pseudoxylaria* and *Termitomyces*, as seen in culture assays (Fig. 4), are a reliable indicator…" (now line 304-306).

Comment 6: Line 292-293: Yet you spend line 293 – 327 defending this controversial hypothesis that your results do not support. Numerous studies have shown that *Pseudoxylaria* and *Termitomyces* thrive in the same growth conditions. This yet again supports the general strategy of *Xylaria* fungi as opportunistic fungi that take over substrate once it becomes available.

Response: We have stated that this view is controversial (now lines 292-293) but that does not mean it is unscientific. We would make the appropriate changes if it were pointed out how and why it is unacceptable. Below we present these lines with our explanations.

	Lines from the Previous Revision	**Sentences**	**Explanation**
1	290-292	*Pseudoxylaria*, with its faster growth and constitutive fungicidal activity (Fig. 4, Supplementary Fig. S13, and Table S8), can be a more efficient inhibitor of non-specific fungi than *Termitomyces*.	Statement based on data generated.
2	292-293	This hints towards its potential beneficial role in this symbiosis, but such a contention remains controversial.	Self-explanatory.

3	293-294	*Pseudoxylaria* has been considered to be an opportunistic fungus which can take over the crop monoculture when conditions are suitable[50].	Statement of fact.
4	294-296	As termites cannot feed on any other fungi[51], the invasion of *Pseudoxylaria* is considered to be indicative of the collapse of this symbiosis[52].	Statement of fact.
5	296-298	However, if the anti-fungal capabilities of *Pseudoxylaria* and *Termitomyces*, as seen in culture assays (Fig. 4), are a reliable indicator of their capabilities within a fungus comb, then it is clear that *Termitomyces* is a less efficient inhibitor of non-specific fungi than *Pseudoxylaria*.	Conjecture based on facts.
6	299-300	This inefficiency can be a selection pressure for the accommodation of *Pseudoxylaria* into this symbiosis.	Conjecture based on facts.
7	300-302	Some evidence towards this is provided by the presence of *Pseudoxylaria* in the core-mycobiota of *O. obesus* (Fig. 2 and Supplementary Table S9) as it argues against its role as an incidental infection[50].	Self-explanatory.
8	302-303	Moreover, its presence in the alates (Fig. 2) also hints at successful transmission across generations.	Self-explanatory.
9	303-304	The extensive taxonomic review of this group[53,54] puts *Pseudoxylaria* to be exclusively associated with Macrotermitinae termites.	Self-explanatory.
10	304-306	Therefore, *Pseudoxylaria* is either an active member of this symbiosis or is an extremely specialized weed or parasite.	Conjecture based on facts.
11	306-308	Second, Fricke et al[55] reported that the genome architecture of *Pseudoxylaria* is consistent with a symbiotic lifestyle as they reveal significantly reduced genome sizes with a loss of lignin metabolizing capabilities.	Statement of fact.
12	308-310	Therefore, this could be indicative of a mutualistic role for *Pseudoxylaria*, involved in preventing other fungi, while	Conjecture based on the above fact.

		simultaneously being dependent on *Termitomyces* for nutrition through lignocellulose digestion.	
13	310-312	Third, Visser et al[50] reported that both *Pseudoxylaria* and *Termitomyces* can use the same Carbon sources to grow, making them strong competitors with the potential of one eliminating the other within a fungus comb.	Statement of fact.
14	312-314	However, as Supplementary figure S9 shows, *Termitomyces*, which has inhibitory effects against other fungi, cannot prevent *Pseudoxylaria* growth and is easily overtaken by it.	Statement of fact.
15	314-316	This inability of *Termitomyces* to prevent *Pseudoxylaria* is contrary to the expected outcome of the evolution of some mutual inhibitory capabilities, especially if they are competing for the same resources[50].	Statement of fact.
16	316-318	However, there are several fungal (Fig. 4) and bacterial strains (Fig. 5)[33] that can do the same and yet a functioning comb is still found to be dominated by *Termitomyces* (Fig. 3).	Statement of fact.
17	318-322	Fourth, a mutualistic role of *Pseudoxylaria* predicts its presence in appreciable amounts even in a fresh comb. As figure 3 indicates, the OTU data barely supports this as only 51 reads identified as *Pseudoxylaria* from the fresh fungus comb. However, qPCR estimates, which are a better estimator of abundance, indicate the presence of $6.0x10^5$ copies (95% confidence intervals; Supplementary Table S6).	Statement of fact.
18	322-324	When taken together, these two estimates prove the presence of *Pseudoxylaria* in fresh combs and are contrary to previous estimates[24].	Conjecture based on facts.
19	324-326	The control of *Pseudoxylaria* can perhaps be enforced by secondary mutualistic bacteria in these colonies, as several such bacterial strains have been identified from these same mounds[33].	Conjecture based on facts.

20	326-327	This suggests a complex interdependent relationship between *Termitomyces, Pseudoxylaria,* and the mutualistic bacteria.	Hypothesis emanating from the above facts.

Comment 6: Comment 22: The fact that you see inhibition of *Termitomyces* even after 36 hours of growth is very interesting and I would suggest you highlight/explore this further.

Response: We thank the reviewer for this. We have added the following lines 513-516:

"Going beyond the 36 hrs of incubation time may not yield accurate results as any inhibition seen can be attributed to the depletion of nutrients from the media or *Termitomyces* dying than to the antifungal activity of *Termitomyces*."

Comment 7: Comment 23: "This essentially means that germination is suppressed by either the termites or by the unfavorable conditions within a comb. The latter seems implausible as the combs have ideal growing conditions for many fungi". More and more studies are highlighting the holistic approach of many symbioses to prevent the growth of antagonists. Within fungus-farming termites it is not only the symbiosis members that are responsible for ensuring the proliferation of *Termitomyces* and inhibition of non-*Termitomyces*. The termite ensures optimal growth condition for *Termitomyces* and the inner environment (high CO2) of the colony itself have been shown to affect the growth and germination of unwanted fungi. Ref: https://doi.org/10.3389/fevo.2023.1134492

Response: This paper shows the presence of relatively high CO_2 concentrations within some African termite mounds and tries to mimic those conditions in culture assays for *Termitomyces*, *Pseudoxylaria* and sundry other pathogenic fungi. It finds that while both *Termitomyces* (weak evidence) and *Pseudoxylaria* (strong evidence) can thrive in those conditions the pathogenic fungi cannot (weak evidence). This indicates that both *Termitomyces* and *Pseudoxylaria* can thrive under similar conditions inside the mound. What has surprised us is the conclusion that the authors draw where this is taken as evidence for the 'sit-and-wait' strategy for *Pseudoxylaria*. If conditions are conducive and both fungi survive on the same finite resources, then both are expected to grow unhindered. Yet, this simple fact is ignored and instead is shoehorned into evidence for the 'sit-and-wait' strategy. Needless to say, we do not agree with the conclusion of this paper and any discussion on it is irrelevant here.

Comment 4, part 4: Your qPCR (of genomic material) and the *in vitro* inhibition assays are showing two very different growth stages of *Pseudoxylaria*. What your growth assay is showing is *Pseudoxylaria's* production of antimicrobial compounds with the ability to limit the growth of non-*Termitomyces* fungi to ensure *Pseudoxylarias* proliferation on the new available substrate. Which is the known strategy of *Xylaria* fungi.

As per my previous comment, your results indicates that the antifungal potential of *Pseudoxylaria* against environmental fungi aids *Pseudoxylaria* in being the fastest to overgrow the newly available nutrient-rich resource once the colony is in decay. From what I can find, this study is the first to test *Pseudoxylaria* antifungal potential against ecological relevant fungal strains isolated directly from fungus combs. This is the novelty of your study.

To sum up: The presence of antimicrobial activity in *Pseudoxylaria* based on the qPCR and the growth assays does not provide compelling enough evidence to suggest a mutualistic role of *Pseudoxylaria* within fungus-farming termites and that this hypothesis that *Pseudoxylaria* aids in keeping the symbiosis free from diseases needs to be reevaluated before the manuscript can be submitted.

Response: We agree with the reviewer that *Pseudoxylaria* can outcompete other fungi when given the same resources. This is precisely what we have observed in our comb decay experiments as well as in our *in vitro* studies. The significance of this phenotype of *Pseudoxylaria* can be viewed in two ways. The first, which seems to be the view of the reviewer, is this phenotype is a 'stand-alone' feature of *Pseudoxylaria* and has nothing to do with preventing other environmental fungi. The second, which we are trying to forward, is a more parsimonious albeit a complex view. Our contention is to view the fungicidal capabilities of *Pseudoxylaria* with respect to the niche of the fungus combs. Therefore, given the high levels of non-specific fungal contaminants, high growth rates of *Pseudoxylaria*, suppressive capabilities of *Pseudoxylaria* extending even against *Termitomyces*, we are proposing that *Pseudoxylaria* can function as a symbiont. We provide evidence to show that it is present in fresh combs but we also hypothesize that it is prevented from growing over or suppress *Termitomyces* by the termites and other bacterial mutualists. The latter accounts for the near-invisibility of *Pseudoxylaria* in fresh combs but is reversed as soon as the termites are removed. This is precisely what we have done to set up our studies. Our assumption that *Pseudoxylaria* continues to grow even in a fresh comb is based on the data that we have obtained. We also did not find any visible *Pseudoxylaria* in fresh combs but obtained this evidence through Nanopore and qPCR data. We have described this in fig 3. The strong antifungal phenotype of *Pseudoxylaria* fits in perfectly with this view. We are hypothesizing that *Pseudoxylaria* continues to prevent other fungi irrespective of whether it is growing extensively or not. This is the reason why we think that *Pseudoxylaria* can be a symbiont in these

termite mounds. Now, if we are to believe the 'sit-and-wait' strategy, unrelated to what the termites are doing, then we have to make many more assumptions. First, we have to assume that *Pseudoxylaria* is absent from fresh combs, which given our data, cannot be true for *Odontotermes obesus*. Second, we have to assume that some unknown force is preventing *Pseudoxylaria* from growing in fresh combs. Third, *Pseudoxylaria* can 'resurrect' itself by 'detecting' the absence of termites and/or comb decay and start proliferating. Fourth, *Pseudoxylaria* can only utilize their antifungal phenotype to "outgrow" and not "prevent" other fungi.

To sum up, our goal here is not to disprove the 'sit-and-wait' or any other strategy but to explain our data in the context of what is known in fungus-growing termites. Therefore, we have presented a model that gives the most parsimonious explanation of our data. As we have repeatedly argued in the previous revisions this model is open to others to validate or reject with data of their own. Therefore, with the data that we have generated we fail to understand why is our view less parsimonious than the 'sit-and-wait' strategy.

We have tried to tone down our conclusions and have added several lines towards that goal. Below we represent that attempt.

1. Lines 324-325: Our toning down of the evidence on *Pseudoxylaria* numbers seen in the fresh comb.

"However, whether these numbers represent actively growing *Pseudoxylaria* or not remains to be seen."

2. Lines 349-360: Our toning down of our conclusions of *Pseudoxylaria* as a symbiont.

"The hypothesis that *Pseudoxylaria* is an additional symbiont still needs to be established as the culture-dependent studies do not replicate the full complexity of these interactions as they occur in nature. Theory suggests that two or more mutualistic partners cooperate and benefit each other without harming the other partner[57,58]. The instances where one partner attempts to exploit the relationship by not reciprocating the benefits leads to the extinction of the mutualism. In this case inhibiting the sole crop of termites, *Pseudoxylaria* does not meet the criteria to qualify for the position of symbiont in this system. The presence of *Pseudoxylaria* in fresh fungus combs might indicate the less harmful role of *Pseudoxylaria* initially for entry into the colony. This can change with the age of the colony where it can overtake the termite garden and behave as a typical parasite or weed of the fungus gardens. Moreover, for *Pseudoxylaria* to be a mutualist it must be present in a fresh fungus comb in appreciable numbers. We have shown that this is true for the two mounds of *O. obesus* used in this study."

3. Lines 360-361: Criteria for proving our hypothesis wrong.

"However, if such further evidence is not obtained from other mounds, then this evidence can be considered as incidental and an artifact of the two mounds used."

4. Lines 362-375: Further toning down of our conclusions of *Pseudoxylaria* as a symbiont.

"Several studies have discussed the coevolution of *Pseudoxylaria* with the fungus-farming termites, yet there still remains some confusion on the ecological role of *Pseudoxylaria* in this symbiosis[51,52]. While some reports propose its role in biomass degradation[38,52], others suggest an antagonistic relationship based on culture dependent studies[53]. Several aspects of *Pseudoxylaria* functioning remains unclear like its role during the formation of a comb as it necessitates the demonstration of the anti-fungal effects of *Pseudoxylaria*[48] even in these early stages. Some of the experiments detailed here are relatively difficult to do because they involve sampling substantial amounts of fungus combs and consequently, only large and mature colonies can be used, which are few and far between. Therefore, these studies need to be confirmed with additional sampling from different fungus-growing termites. Thus, the role of *Pseudoxylaria* in fungus-termite system is mostly considered that of a weed and threat to termite colony unless concrete evidence emerges to the contrary. Therefore, our verbal model, awaits empirical validation as future studies with more mounds and different species of termites are needed to establish whether this model is validated or the role of *Pseudoxylaria* remains that of a weed and/or parasite."

REVIEWERS' COMMENTS:

Reviewer #3 (Remarks to the Author):

The updated version of the manuscript adequately considers all suggestions made by reviewer 4. The authors' reply also address all concerns raised by that reviewer. As I stated in my previous review, this manuscript brings an alternative view on how to interpret (or understand) the role of Pseudoxylaria in termite combs. As scientists we should always provide alternative explanations for phenomena but only based on evidence. The proposed model in this manuscript is based on evidence derived from the obtained data and after this long review process, authors are interpreting this model considering all caveats it brings along. Thus, I'm happy with this new version of the manuscript and the authors' reply. I also found this manuscript easy to read. I congratulate the authors for the clear thought put into words in this manuscript.

I do have minor edits on the manuscript and one comment to consider in the model.

Food for thought comment:

L. 279-281: "This can be indicative of a dynamic equilibrium where the growth of Pseudoxylaria is tolerated by the termites to some extent, perhaps to prevent other non-specific fungi, but is also simultaneously inhibited from taking over the comb."

I understand the point the proposed model brings: Pseudoxylaria, at least at early stages of comb decay (or even in fresh comb), might act as a beneficial symbiote using the antifungal arsenal suppressing non-specific fungi. As shown in vitro assays, Pseudoxylaria produces antifungals with a broad range of activity on several non-specific fungi. However, how does the model hold as a proposal for testing as Pseudoxylaria-derived antifungals inhibit Termitomyces? Clearly, this happens as shown in Fig. S12. Thus, even under a "dynamic equilibrium", how would Pseudoxylaria antifungals selectively target non-specific fungi but not Termitomyces? The proposed model does not consider this important fact derived from the data.

Minor comments:

L. 25 and throughout: One concern I raised in my previous review is in regards the expression "bacterial mutualists" in this manuscript. How does one know these bacteria are indeed mutualists? To determine if they are, one should assess the reciprocal benefits of both partners on their fitnesses. For the termite side, bacteria found in this study protect against non-specific fungi. However, what are the benefits for the bacteria? This is hard to assess as this need experiments targeted to answer this question. My suggestion is to use the word "symbiotes" instead of "mutualists", following the concept described in https://doi.org/10.1016/j.funeco.2019.100909

L. 59 "…microfungi, yeasts, and bacteria.": I believe a yeast is also a microfungus. Perhaps, use "microfungi and bacteria".

L. 187: Figure S8 does not compare Termitomyces, Pseudoxylaria and other non-specific fungi. Thus, the statement "It appears that the abundances of these two fungi are negatively correlated with the presence of other non-specific fungi " is not supported by data presented in Fig S8. Please, have a careful look at all citations of supplementary figures as it appears they are misplaced throughout the main text.

L. 190-191: same issue here.

L. 336: I believe “parasitize” is not the correct word here. Perhaps, “compete” seems appropriate as this manuscript also emphasizes that the role of Pseudoxylaria is controversial and parasitism could be one side of the story. To be “neutral” in this controversy, perhaps “compete” seems appropriate.

L. 766-768: Legend of Figure 6: This legend should explain the model in full for two reasons: First, the model is complex and the need to go back and forth in the discussion and the figure makes it even more difficult to follow. Second, people only skimming the paper may get a wrong impression of the message this model wants to deliver.

Response to reviewer #3

We thank reviewer #3 for the re-assessment of our manuscript. Below, we present a detailed response to the reviewers' comments:

Reviewer 3, general comment: L. 279-281: "This can be indicative of a dynamic equilibrium where the growth of *Pseudoxylaria* is tolerated by the termites to some extent,perhaps to prevent other non-specific fungi, but is also simultaneously inhibited from taking over the comb." I understand the point the proposed model brings: *Pseudoxylaria*, at least at early stages of comb decay (or even in fresh comb), might act as a beneficial symbiote using the antifungal arsenal suppressing non-specific fungi. As shown in vitro assays, *Pseudoxylaria* produces antifungals with a broad range of activity on several non-specific fungi. However, how does the model hold as a proposal for testing as *Pseudoxylaria*-derived antifungals inhibit *Termitomyces*? Clearly, this happens as shown in Fig. S12. Thus, even under a "dynamic equilibrium", how would *Pseudoxylaria* antifungals selectively target non-specific fungi but not *Termitomyces*? The proposed model does not consider this important fact derived from the data.

Response: We have indicated this caveat in several places in the discussion. These are lines 258-260, 296 and almost the entire last paragraph of the discussion (lines 349-381). We have speculated how such a protection can be achieved in lines 281-291, 305-309, 322-324, 353-354 and 363-365. However, we do admit this remains a major drawback of our model. We think that the termites might hold the key as the selection pressure on them should be strong enough to find a solution. As our study began after removing termites this aspect remains unknown. However, we have added the following lines (358-363):

'As our study used fresh and mature combs, the role of *Pseudoxylaria* in the early stages of the comb formation, especially how a growing culture of *Termitomyces* remains immune from the generic antifungal properties of this weed remains unknown. Moreover, as our study also viewed the microbial dynamics after removing the termites, their role in defending *Termitomyces* against *Pseudoxylaria* is also undeciphered. Clearly, future studies on these fungi must involve all partners, especially in incipient combs, to answer some of these questions.'

Minor comments:

Reviewer 3, comment 1: L. 25 and throughout: One concern I raised in my previous review is in regards the expression "bacterial mutualists" in this manuscript. How does one know these bacteria are indeed mutualists? To determine if they are, one should assess the reciprocal benefits of both partners on their fitnesses. For the termite side, bacteria found in this study protect against non-specific fungi. However, what are the benefits for the bacteria? This is hard to assess as this need experiments targeted to answer this question. My suggestion is to use the word "symbiotes" instead of "mutualists", following the concept described in https://doi.org/10.1016/j.funeco.2019.100909

Response: We have changed the 'bacterial mutualists' to 'bacterial symbiotes' and 'mutualistic bacteria' to 'symbiotic bacteria'.

Reviewer 3, comment 2: L. 59 "…microfungi, yeasts, and bacteria.": I believe a yeast is also a microfungus. Perhaps, use "microfungi and bacteria".

Response: We have removed the yeast from the statement (Line 59).

Reviewer 3, comment 3: L. 187: Figure S8 does not compare *Termitomyces*, *Pseudoxylaria* and other non-specific fungi. Thus, the statement "It appears that the abundances of these two fungi are negatively correlated with the presence of other non-specific fungi " is not supported by data presented in Fig S8. Please, have a careful look at all citations of supplementary figures as it appears they are misplaced throughout the main text.

Response: We had uploaded an older version of the supplementary file by mistake. We apologize for this and have now corrected it.

Reviewer 3, comment 4: L. 190-191: same issue here.

Response: We have updated the supplementary file.

Reviewer 3, comment 5: L. 336: I believe "parasitize" is not the correct word here. Perhaps, "compete" seems appropriate as this manuscript also emphasizes that the role of *Pseudoxylaria* is controversial and parasitism could be oneside of the story. To be "neutral" in this controversy, perhaps "compete" seems appropriate.

Response: We have changed the 'parasitize' to 'compete' (Line 337).

Reviewer 3, comment 6: 766-768: Legend of Figure 6: This legend should explain the model in full for two reasons: First, the model is complex and the need to go back and forth in the discussion and the figure makes it even more difficult to follow. Second, people only skimming the paper may get a wrong impression of the message this model wants to deliver.

Response: We have changed the legend of Figure 6 to:

"A proposed model for understanding the interactions among the bacteria, fungi and termites in *O. obesus* colonies to maintain weed free agriculture of *Termitomyces*. It suggests a possible mutualistic role of the weed, *Pseudoxylaria,* as it can control the growth of many non-specific fungi brought in by the termites. It also proposes that *Pseudoxylaria,* which can overgrow *Termitomyces,* is kept in control by bacterial symbiotes in fresh fungus combs. The non-specific fungi that compete for resources can also be inhibited by the *Termitomyces* and bacterial symbiotes. These interactions suggest a complex network of microbial management, and is important for understanding the role of *Pseudoxylaria* within these termite colonies."