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Microbial studies on *Odonotermes SPP* termites in three different areas of Pollachi, Coimbatore, Tamil Nadu

Jiny Thomas, Rosaline Mary
Department of zoology, Nirmala college for women, coimbatore, Tamil Nadu, india

Abstract
Termites are important insects for the environment because they break down organic materials and recycle nutrients. Despite their significance, little is known about the intricate link between termites and their gut microbiota. With an emphasis on nutrient cycle and decomposition processes, this study explores the symbiotic interactions between termites and the microorganisms that are connected with their gut. We discovered important microbial taxa involved in nitrogen fixation and lignocellulose breakdown by characterising the stomach microme of three termite species using a mix of genetic and culture-based approaches. Our findings show a complicated web of relationships between termites, their intestinal diet, and the health of the ecosystem. This study advances our knowledge of termite biology and has ramifications for the creation of innovative biotechnology uses.

Keywords: Termites, symbiotic relationship, decomposition, microme, gut

Introduction
Termites are crucial to ecosystems because they have a significant impact on the physical and chemical makeup of soil, plant degradation, the cycling of carbon and nitrogen, and microbial activity. In the tropics, termites are the most significant invertebrate decomposers, whereas in temperate regions, they have a small ecological impact (Bignell *et al*., 2011).

A vast variety of life forms can be found in soil, making it one of the most intricate and species-rich environments. Termites establish eusocial communities, live in colonies, and build arboreal, epigeous, or subterranean nest systems. Subterranean, dry-wood, and damp-wood termites are the three broad categories into which termites can be divided according to their habitat (Paul *et al*., 2005). Both soil and timber that comes into touch with soil are home to subterranean termites. Their intense requirement for moisture in their surroundings, which they meet by nesting inside or in close proximity to the soil, gives them the moniker "subterranean" (Thorne, 1998).

A pair of alates (winged termites), the prime reproductives that create all of the nestmates, often establish a termite colony. According to Haifig *et al*. (2016), secondary reproduction appears to either supplement or replace primary reproduction in certain species. The members of termites' intricate society are divided into various castes based on their morphology, physiology, and behaviour.

According to conventional ranking, termites belong to the insect order Isoptera, which is a subgroup of Blattodea. Their sister taxon is *Cryptocercus* (Lo *et al*., 2000).

Dead wood is the primary food source for lower termites. Instead of spreading vertically (from mother to child), the symbionts establish themselves gradually, giving the offspring access to the majority of the microbiota before workers emerge. This is likely due to social interactions with nursing staff (Diouf *et al*., 2015).

Research Gap
The study's comprehension of the isolated bacteria's cellulolytic ability is limited because it doesn't look into the genetic and molecular pathways underlying their destruction of cellulose. Understanding of the isolated bacteria's potential use in biomedicine is limited because the study does not look into their possible antibacterial efficacy against a variety of pathogens. Ahmad Bashir *et al*. (2013) [2]

Formulation of the Problem
We hope to aid in the creation of long-term solutions for environmental problems by discovering and describing these bacteria. Termites live in a variety of habitats with different climates, food, and species, all of which might affect the gut microbiota's makeup and capacity to break down cellulose. By collecting termites from various habitats and geographical areas, we hope to investigate environmental and regional differences in cellulose-degrading bacteria, improving our knowledge of microbial ecology and adaptation.

Materials and Method
Sterilization and Dissection of Termites
Termites are gathered and placed in plastic jars, which are subsequently sterilised to shield them from the microbes they come into contact with while travelling. 90% ethanol is used to sterilise the termites' surfaces in order to accomplish this. After being surface sterilised, the particular termite samples are dissected using a sterilised scalpel blade that has been subjected to UV light for 15 minutes to remove any possible impurities. By reducing the possibility of introducing foreign bacteria during the dissection process, this meticulous technique guarantees the preservation of the research integrity and makes it easier to accurately analyse and investigate the termites' microbiomes. The bacteria in the stomach is what breaks down cellulose during this dissection process since the head, limb, and other unneeded body parts are cut off, leaving only the gut intact. the crucial step prior to vaccination.

Termite – *isopteran*

Inoculation

Once the termite has been dissected, the head, limbs, and any extraneous components should be removed. The Mandel basal media is a broth into which six worker termites are introduced. 1.0 g/L MgSO47H2O, 2.0 g/L KH2PO4, 3.5 g/L peptone, 3.0 g/L yeast extract, and 1.0 g/L (NH4)2SO4 make up the medium. Additionally, the only carbon source is either Birchwood xylan or 10.0 g/L of carboxymethyl cellulose (CMC). After adjusting the medium's pH to 7.0, it is incubated for 24 hours at 37°C and 150 rpm (Mandels, 1969). The six worker caste termites are injected into 100 mL of Mandel basal medium after it has been prepared and autoclaved. As a control, a different broth is also made without termite inoculation. To verify whether the bacteria have been developed, termites from three distinct regions are cultivated in various broths alongside a control.

Zone of Clearance

A quick and accurate way to screen for cellulolytic bacteria is to use Congo Red as an indicator for cellulose degradation in an agar medium (Wang *et al*., 2004). Bacteria that exhibit Congo Red discolouration are recognised as positive cellulose-degrading bacteria, demonstrating their capacity to break down cellulose. These colonies are the only ones chosen for additional research. 0.2g of Congo Red, 0.5g of LB-broth, and 2g of carboxymethyl cellulose (CMC) are combined in 100ml to create the agar medium. It is then autoclaved and transferred into sterile plates to cool. After that, the agar is split into four wells: three for the samples and one for the control. Each well is inoculated with 100 µl of bacterial culture using sterile tips, and the plates are then incubated for a full day. A apparent colour shift in the area around the wells following incubation is a sign of cellulose degradation because the enzymes secreted by cellulolytic bacteria break down cellulose and break the bond between Congo Red and cellulose.

Serial Dilution

In bacterial culture, serial dilution broth entails moving a part of the culture into a series of dilution tubes filled with broth or sterile water in order to gradually lower the bacterial concentration. This procedure is essential for a number of reasons, including the fact that it dilutes the culture to a tolerable concentration for precise colony counting, which makes it possible to count live bacteria. It facilitates colony enumeration by guaranteeing the development of distinct colonies on agar plates. Lastly, it enables scientists to create bacterial cultures at precise concentrations that are appropriate for a range of experimental uses, including biochemical tests and inoculation. After dissolving 1g of carboxymethyl cellulose (CMC) in 100ml of distilled water, the mixture was autoclaved to remove any remaining contaminants. Nine millilitres of a 1% CMC solution were then transferred into eight test tubes. The first test tube was then injected with a 1 ml aliquot of bacteria that had been pipetted out of the broth. One millilitre was moved from the first test tube to the second, and so on, until the seventh test tube was reached. By lowering the bacterial load in the culture, this process enables more carefully monitored experiments.

Isolation of Pure Culture

A sample with a bacterial concentration of 10^7 CFU/mL is collected from test tube number 7 after the serial dilution procedure is successfully finished. After that, 100 µl of the contents of this test tube are transferred onto a Congo Red agar plate that has cellulose as its only carbon source. The spilt bacterium is then disseminated with a L rod. To enable bacterial growth, the plate is incubated for a full day. Following incubation, bacterial colonies that are encircled by an obvious agar colour shift that signifies cellulose breakdown are recognised and labelled. A tiny amount of bacterial growth is transferred from the agar plate into a broth containing cellulose in a boiling tube using a sterile toothpick or inoculation loop in order to separate pure cultures from these colonies. To track the growth of bacteria, the boiling tube is then incubated for a further twenty-four hours.

Catalase Test

Based on their capacity to produce the catalase enzyme, bacteria can be identified using the catalase test, which functions similarly to a detective tool. An enzyme called catalase aids in the conversion of hydrogen peroxide into oxygen and water. Bacteria that produce catalase will bubble when exposed to hydrogen peroxide because oxygen gas is released. We witness this bubbling reaction. The presence or lack of catalase can aid in the differentiation of different bacterial species based on their distinct metabolic capacities. For instance, while *Streptococcus species* do not manufacture catalase, many common bacteria, such as *Staphylococcus species*, do. Therefore, if hydrogen peroxide is added to the test tube and bubbling is seen, the result is positive and the bacteria are producing catalase. A negative result indicates the lack of catalase synthesis if there is no bubbling. Two millilitres of bacterial culture are transferred to a test tube, and two millilitres of hydrogen peroxide are added. If bubbling is observed, the sample is producing the catalase enzyme. Otherwise, it is regarded as a bad outcome.

Gram Staining

A basic method in microbiology, Gramme staining divides bacteria into two major groups, Gram-positive and Gram-negative, according to the makeup of their cell walls. For preliminary bacterial identification, this staining technique is crucial. There are usually multiple steps in the process. First, a bacterial culture is spread out onto a microscope slide and left to air dry to create a bacterial smear. In order to prevent deformation during staining, the slide is then heat-fixed to firmly bind the germs and gently kill them. A main stain called crystal violet is then poured across the slide, giving all of the bacteria a purple hue. Water is used to clean away excess stain. Iodine solution, which serves as

a mordant to improve the stain's adherence to the bacterial cells, is next poured onto the slide. The iodine solution is removed after a short while, and acetone or alcohol is used to rinse the slide. Decolorisation is the process that distinguishes between Gram-positive and Gram-negative bacteria. Gram-negative bacteria lose the stain and turn colourless, but Gram-positive bacteria keep the crystal violet-iodine complex and look purple. Gram-negative bacteria are stained pink or red when the slide is counterstained with a contrasting dye, usually safranin. Before being examined under a microscope, excess counterstain is wiped off and the slide is allowed to air dry.

Methyl Red Test

This test evaluates the bacteria's capacity to ferment glucose and create stable acids. When fermenting glucose, it's especially helpful in distinguishing between bacteria that create mostly neutral products (like *Enterobacter aerogenes*) and those that produce mixed acids (like *Escherichia coli*). The Methyl Red indicator dye is introduced after a bacterial culture has been inoculated into MR Broth, which contains glucose. The medium's pH falls and the Methyl Red dye turns red if the bacteria create stable acids as byproducts of fermenting glucose. This implies that the bacteria can ferment mixed acids, which is a positive test result. A negative test is produced if the bacteria create weak acids or neutral compounds, which keeps the pH largely constant and prevents the Methyl Red dye from turning red. A positive result (showing mixed acid fermentation) is indicated by the colour red, whereas a negative result (meaning neutral fermentation or weak acid generation) is indicated by the colour staying yellow or orange.

Vogues-Proskauer Test

This test is used to identify the presence of acetoin, a metabolic byproduct that some bacteria, especially those in the Enterobacteriaceae family, produce during fermentation. Alpha-naphthol and potassium hydroxide reagents are added after a bacterial culture has been inoculated into VP Broth, a broth made with glucose and peptone (1g of peptone and 0.5g of glucose in 100ml of distilled water). A red colouration that indicates the presence of acetoin indicates a good result, whereas little to no colour change indicates a negative result. While organisms like *Shigella species* and *Escherichia coli* frequently produce unfavourable results, bacteria like *Klebsiella pneumoniae* and *Enterobacter aerogenes* are usually linked to positive outcomes.

The biochemical tests used in this research paper to identify the bacteria were the catalase test, which produced a positive result, the starch hydrolysis, which also produced a positive result, and the Gramme staining, which also produced a positive result. We performed a biochemical test on a pure bacterial culture of bacteria that we recovered from several regions (Kakara *et al*., n.d.) [35]. Using 16s rRNA sequencing, we were able to identify the bacterial strains and obtain positive results for starch hydrolysis and the catalase test. For each of the 11 bacterial strains that we isolated and found to be capable of breaking down cellulose and CMC, we performed a catalase test. We also performed starch hydrolysis, and the findings were both positive and negative. Then, in order to describe and comprehend the fermentation route, we performed a glucose fermentation test.

The Lowry method is used to quantify the total protein concentration first, and then an enzyme test is conducted to measure the activity of endoglucanase and exoglucanase (total cellulose). Fresh cellulolytic bacterial strains' total cellulase activity was measured, characterised, and expressed in filter paper units (FPU) per millilitre of undiluted culture filtrate. The amount (mg) of reducing sugar released per hour under conventional test conditions (540 nm) is known as one FPU.We haven't determined the protein concentration, which is necessary to determine the enzyme activity and particular enzyme activity (Kakara *et al*., n.d.) [35]. Future calculations of enzyme activity would be made if the Lowry technique could be used to measure protein content.

Catalase test

I: No	1	2	3	4	5	6	7	8	9	10	11
SA	++	-	++	+	++	+	+++	+	+	++	+++

Gram staining

I: No	1	2	3	4	5	6	7	8	9	10	11
SA	+	-	+	+	+	-	+	+	+	+	+

Methyl red test

I: No	1	2	3	4	5	6	7	8	9	10	11
SA	+	+	-	-	-	-	-	+	+	-	+

Vogues-Proskauer test

I: No	1	2	3	4	5	6	7	8	9	10	11
SA	++	++	++	+	+	+	-	+	+	-	+

Carboxymethy cellulase Assay

Isolate No:	Catalase test	Gram staining	Methyl red test	Vogues-Proskauer test
1	++	+	+	++
2	-	-	+	++
3	++	+	-	++
4	+	+	-	+
5	++	+	-	+
6	+	-	-	+
7	+++	+	-	-
8	+	+	+	+
9	+	+	+	+
10	++	+	-	-
11	+++	+	+	+

Discussion

For the purpose of producing enzymes, eight positive isolates (CDB1, 2, 3, 6, 7, 8, 9, and 10) were chosen, and each one's cellulolytic activity was calculated. In the enzyme experiment, CDB 10 had the highest cellulase activity on filter paper (0.194 IU/mL), while CDB 8 had the highest endoglucanase activity (0.400 IU/mL). The FPCase and endoglucanase assays showed activity ranging from 0.012 to 0.196 IU/mL and 0.1622 to 0.400 IU/mL, respectively. (Gupta and others, 2012) [26]. The zone of clearing in Congo red media with carboxymethyl cellulose (CMC) as the only carbon source verified that we had isolated 11 pure bacterial strains that could break down cellulose. We next performed a DNS experiment and measured the absorbance of the crude enzyme that the bacteria produced in a UV spectrophotometer. The release of more reducing sugars is indicated by a higher absorbance.

At 540 nm, isolate number 2, which was determined to be Paracoccus yee, had an absorbance of 0.219, whereas Lactococcus nasutitermitis had an absorbance of 0. 189.By determining the protein content, we could determine the specific enzyme activity for the detected isolate. After determining the precise enzyme activity, we may determine the Kmax and Vmax, which represent the enzyme kinetics. Finding the following factors would allow us to compare our cellulase enzyme with those that are sold commercially and determine which is the best. We can commercialise our cellulase enzyme if it exhibits higher activity than the commercially available cellulase.

Nine species of Australian termites from four families had their primary gut bacteria extracted and identified to a generic level. Every species was either a strict aerobe or a facultative anaerobe. There seems to be a relationship between the termite's family and the main gut bacterium. Streptococcus was the predominant bacterium from the two lowest termites, Mastotermes darwiniensis (family Mastotermitidae) and Cryptotermes primus (family Kalotermitidae); Enterobacter was found in four Rhinotermitidae species (Heterotermes ferox, Coptotermes acinaciformis, C. lacteus, and Schedorhinotermes intermedius intermedius); and Staphylococcus was found in three Termitidae species (Nasutitermes exitiosus, N. graveolus, and N. walkeri).We used termites from the Tamil Nadu district of Thanjavur, whereas Eutick *et al*. (1978) [22] used termites from Australia. Following 16s rRNA sequencing of two bacterial stains from 11 pure stains, we were able to identify Paracoccus yee and Lactococcus nasutitermitis based on the results of the biochemical test and CMC assay.

Cellulolytic, xylanolytic, and ligninolytic bacteria were screened using the Mandel basal solid medium supplemented with 10 g/L of CMC, 10 g/L of birchwood-xylan, or 2.5 g/L of alkaline lignin, respectively. A prefabricated hole in the middle of CMC agar plates, birchwood-xylan agar plates, and alkaline lignin agar plates was infected with 5 lL of bacterial cultures and fungal spores. For 72 hours, the plates were incubated at 37 C. Following 72 hours, 0.1% (w/v) Congo red was added to the CMC plates, and then 1 M NaCl was used to destain them; 0.25 percent (w/v) aqueous I2 and KI were added to the birchwood-xylan plates; and 1% (w/v) aqueous solution of FeCl3 and K3- [Fe (CN)6] was added to the alkaline lignin agar plates. In 2016, Kamsani *et al* [36]. After inoculating bacteria in wells and incubating them for 24 hours, we obtained a zone of clearing, which indicates the presence of cellulose-degrading bacteria, and used Congo red agar media made of a single carbon source as CMC to screen cellulolytic microorganisms.

The biochemical tests used in this research paper to identify the bacteria were the catalase test, which produced a positive result, the starch hydrolysis, which also produced a positive result, and the Gramme staining, which also produced a positive result. We performed a biochemical test on a pure bacterial culture of bacteria that we recovered from several regions (Kakara *et al*., n.d.) [35]. Using 16s rRNA sequencing, we were able to identify the bacterial strains and obtain positive results for starch hydrolysis and the catalase test. For each of the 11 bacterial strains that we isolated and found to be capable of breaking down cellulose and CMC, we performed a catalase test. We also performed starch hydrolysis, and the findings were both positive and negative. Then, in order to describe and comprehend the fermentation route, we performed a glucose fermentation test.

The Lowry method is used to quantify the total protein concentration first, and then an enzyme test is conducted to measure the activity of endoglucanase and exoglucanase (total cellulose). Fresh cellulolytic bacterial strains' total cellulase activity was measured, characterised, and expressed in filter paper units (FPU) per millilitre of undiluted culture filtrate. The amount (mg) of reducing sugar released per hour under conventional test conditions (540 nm) is known as one FPU.We haven't determined the protein concentration, which is necessary to determine the enzyme activity and particular enzyme activity (Kakara *et al*., n.d.) [35]. Future calculations of enzyme activity would be made if the Lowry technique could be used to measure protein content.

Conclusion

We gathered termite samples from the Thanjavur district's different areas. To ensure darkness, these samples were placed in plastic crates and brought to our lab wrapped in black paper. Once they arrived, we sterilised them by surface sterilising them with 90% ethanol. After sterilising the termites, we dissected them, cutting off any extraneous components like the worker termites' head and limbs. We next let the dissected samples to incubate for 24 hours after inoculating them onto Mendal's basal media. By performing a zone of clearance experiment in Congo red medium with cellulose as the only carbon source during the incubation period, we confirmed the existence of cellulose-degrading bacteria. The existence of microbes that break down cellulose was verified by this experiment. We carried out successive dilutions up to 10^7 dilutions in order to lower the bacterial load. We performed spread plate experiments on Congo red medium using the 10^7 dilution. To create pure cultures, bacterial colonies exhibiting colour changes in the media were chosen and subcultured. We performed a number of biochemical assays to distinguish between the separated strains in order to get pure cultures. The reducing sugars produced during the hydrolysis of cellulase enzymes as carboxymethyl cellulose (CMC) were then measured using the DNS assay. Our findings show a strong relationship between absorbance measurements and the reaction mixture's lowering sugar concentration. Greater cellulase activity is indicated by higher absorbance readings, which are a measure of the amount of reducing sugars present. Out of all our pure samples, pure culture sample 2 has the greatest absorbance, followed closely by isolate number 11 with a similarly high absorbance. In order to identify the related bacteria, these two samples are chosen for 16S rRNA sequencing. The study's overall conclusions support the idea that the termite gut's cellulolytic bacteria generate the cellulase enzymes needed to break down cellulose. The bacteria that cause this degrading process have been identified by 16S rRNA sequencing as Paracoccus yee and Lactococcus nasutitermitis. Using the CMC assay, we obtained absorbance values of 0.219 and 0.189 at 540 nm for Paracoccus yee and Lactococcus nasutitermitis, respectively. demonstrating that higher absorbance values correspond to higher concentrations of reducing sugars.

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