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Characterization of Gut Bacterial Diversity in *Odontotermes horni* (Wasmann) : A Study of Symbiotic Microbiota

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Abstract

Termites are crucial ecosystem engineers that play an essential role in decomposing plant material and recycling nutrients. The present study focuses on the gut bacterial communities of *Odontotermes horni.* The bacterial symbionts were cultured on nutrient agar media and molecular methods were employed for their identification. A total of 10 bacterial isolates were obtained from the termite gut, with 80 per cent identified as Gram-negative and 20 per cent as Gram-positive. The majority of isolates belonged to the orders Enterobacterales (50%) and Pseudomonadales (30%), while Bacillales represented 20 per cent of the isolates. At the family level, Enterobacteriaceae (50%) was the most prevalent, followed by Moraxellaceae (30%) and Staphylococcaceae (20%). Key species included *Citrobacter freundii*, *Proteus mirabilis*, *Trabulsiella odontotermitis*, *Escherichia fergusonii* and *Acinetobacter* spp., which are known for their roles in lignocellulose degradation, nitrogen fixation and detoxification of plant-derived compounds.These findings provide insights into the diversity and taxonomic composition of gut-associated bacteria in *O. horni*, underscoring the presence of key bacterial taxa potentially involved in various functions inside the termite gut. This study contributes to a broader understanding of termite-microbe symbiosis and offers a basis for future research into the ecological and metabolic functions of these bacterial communities.

Keywords : Termite, *Odontotermes horni*, Gut bacteria, Enterobacterales

Insects host a wide variety of symbiotic microorganisms, which play a key role in their development and survival (Devaiah *et al.,* 2022 and Pujar *et al.,* 2023). Termites, eusocial insects known for their ability to break down lignocellulosic materials, harbour a remarkably diverse and intricate microbial community within their digestive tracts, which is essential for the host's nutrition and survival (Engel *et al*., 2009 and Adams & Boopathy, 2005). Termites, especially those from the *Odontotermes* genus, are crucial decomposers in tropical and subtropical ecosystems, owing to their ability to degrade lignocellulosic plant material. This process is primarily facilitated by their symbiotic relationships with a complex gut microbiota. The gut microbiota, including bacteria, archaea and fungi, work in tandem to digest cellulose and hemicellulose, allowing termites to derive essential nutrients from wood and plant debris (Brune, 2014).

Fungus-growing termites such as *Odontotermes horni* exhibit a mutualistic relationship with both fungi and a specialized bacterial community residing in their gut. These bacteria not only assist in lignocellulose digestion but also contribute to nitrogen fixation and nutrient cycling (Engel and Moran, 2013). Previous

studies on the gut microbiome of other termite species, such as *Macrotermes gilvus* and *Odontotermes formosanus*, have revealed a diverse bacterial community that plays a pivotal role in termite biology, particularly in lignin breakdown and carbohydrate metabolism (Boucias *et al*., 2013).

Despite the well-documented symbiotic relationship between termites and their gut bacteria, limited research exists on the specific bacterial diversity within *O. horni*. Understanding the bacterial composition in this species could provide a key insight into the role of microbes in the termite's ability to degrade lignocellulose and thrive in nutrient-poor environments (Otani *et al*., 2014). Furthermore, a detailed characterization of the gut bacterial community could have implications for industrial applications in biomass conversion and bioenergy production (Scharf, 2020).

Material and Methods

Sample Collection and Processing

Termite Specimen Collection : Samples of *Odontotermes horni* were collected from Chamundi hills (Latitude: 12º61'51'' N, Longitude: 76º41'20'' E) and K M Doddi (Latitude: 12º31'7'' N, Longitude: 77º1'58'' E) as a part of the study of gut microbial diversity of termites. Termites were transported to the laboratory in sterile plastic containers to minimize environmental contamination. Worker castes were used for bacterial isolation due to their foraging activities, within 24 h after sampling and also for molecular confirmation. Soldier castes were stored in 70 per cent ethanol and utilized for morphology-based termite identification.

Gut Dissection : Termites were surface-sterilized by washing in 70 per cent ethanol and then rinsed with sterile water twice. The entire gut was dissected from three to four termites using sterile forceps, needle and scissors (Fig. 1). Gut samples were pooled to ensure a sufficient amount of material for microbial analysis.

Isolation and Purification of Bacteria

The dissected gut of *O. horni* was homogenized in a microcentrifuge tube using a sterile micropestle with 1 ml of phosphate buffer saline (PBS) solution (pH 7.4). To isolate individual bacterial colonies, serial dilutions of the homogenate were prepared up to 10^{-7}.

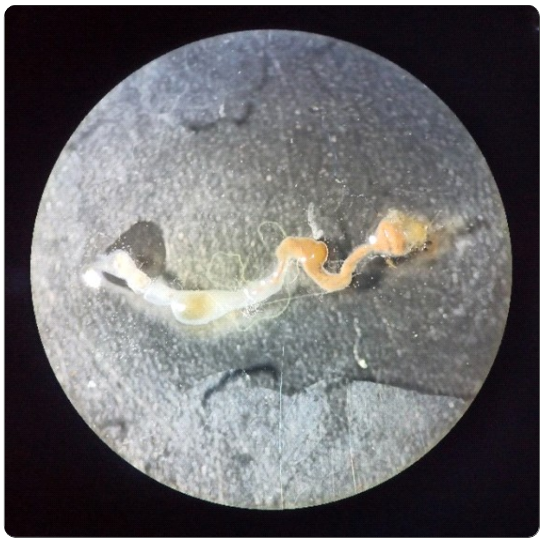

Fig. 1 : Dissected termite gut

From each dilution, 100 µl aliquots were plated onto nutrient agar (NA) using the spread plate technique with a sterilized glass spreader. The plates were incubated at 28°C for 48 hours. This incubation period allows for the development of visible colonies from individual bacterial cells. After every 24 hours, plates were observed for microbial growth. Distinct colonies exhibiting unique morphological characteristics were selected and subcultured onto fresh nutrient agar plates to obtain pure cultures (Fig. 2). For bacterial multiplication, the pure cultures were inoculated into nutrient broth and incubated at 37°C overnight to achieve sufficient biomass for downstream analyses.

Molecular Identification of Gut Bacteria

Total DNA was extracted from isolated pure culture by inoculating a single colony into nutrient broth and incubating at 37°C for 24 hours. After incubation, 1.5 ml of the culture was transferred to a microcentrifuge tube, centrifuged at 10,000 rpm for 3 minutes and the pellet was collected. The

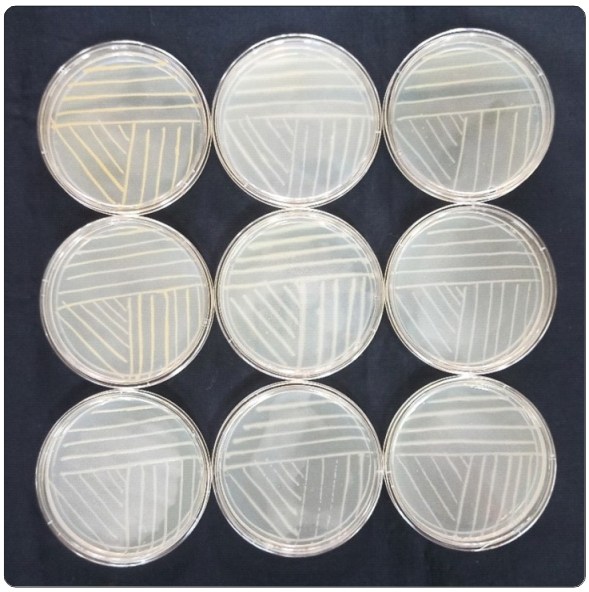

Fig. 2 : Bacterial pure culture plates

pellet was suspended in 400 µl sucrose buffer and vortexed. Later, 32 µl of lysozyme was added and the mixture was incubated for 10 minutes at 60°C. Following this, 45 µl of 10 per cent SDS and 5 µl of proteinase were added, mixed well and incubated in a water bath for another 10 minutes at 60°C. Then, 240 µl of NaCl and 140 µl of freshly prepared 10 per cent CTAB were added and the mixture was kept in the water bath for 10 minutes. Afterward, 500 µl of chloroform: isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phase containing the DNA was transferred to a new tube and 50 µl of 3M sodium acetate and 300 µl of isopropanol were added. The solution was gently mixed and incubated overnight at -20°C. The mixture was then centrifuged at 12,000 rpm for 15 minutes to pellet the DNA. The supernatant was discarded and the pellet was washed twice with 1 ml of 70 per cent ethanol, spinning at 12,000 rpm for 10 minutes each time. After discarding the ethanol and allowing the pellet to dry, the DNA was suspended in 40 µl of TE buffer and 2 µl of RNase was added. The sample was incubated at 37°C for 30 minutes (Swathi *et al*., 2015).

16s rRNA Gene Amplification

The 16S rRNA gene was amplified from bacterial colonies by PCR, using universal eubacterial primer (Table 1). Agarose gel electrophoresis was performed and the aliquots of each PCR product were resolved electrophoretically on 1-1.5 per cent agarose gel using 1XTAE buffer. The PCR products were visualized with a UV transilluminator and photographed with a gel documentation system (Gel Doc 200, BIO-RAD, USA) after staining the gel with ethidium bromide (0.5 µg ml^{-1}) (Promega), the DNA molecular weight marker, a 1-kbp DNA ladder (Promega), was used to determine the size of the amplified fragments. The amplicons were eluted and sent for sequencing. The obtained sequences were analyzed along with the 16S rRNA gene sequences retrieved from the NCBI GenBank using bioinformatic softwares, Bioedit, Clustal W, MEGA XI, *etc*.

Identification of Termite Species

Soldier caste was used for the morphological identification. Species level identification requires measurements of different body parts. For accurate measurement, the alcohol preserved specimens were straightened prior to measurement. A special arena/ platform was prepared in order to stretch the specimen properly and taken the measurements using an ocular micrometer. Fauna of India and the adjacent countries, Isoptera (Termites) Volume II (Family-Termitidae) by Chhotani (1997) was used for the identification of termite.

TABLE 1

Sequence and length of primer used for amplification of 16s rRNA gene

Primer	Sequence	Length
16S rRNA (Forward)	5'-AGAGTTTGATCCTGGCTCAG-3'	20
16S rRNA (Reverse)	5'-ACGGCTACCTTGTTACGACTT-3'	21

Molecular Confirmation of Termite Species

DNA was extracted from 3-5 termite workers using the CTAB method (Doyle & Doyle, 1987). Samples were homogenized in 200 µl CTAB extraction buffer (10 mM Tris-HCl, pH 8; 20 mM EDTA; 1.4 M NaCl; 2% CTAB), followed by addition of 20 µl protease and incubation at 60°C for 10 minutes. After cooling, an equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture was centrifuged at 10,000 rpm for 10 minutes. The aqueous phase was then transferred, mixed with 0.7 volume of ice-cold isopropanol and stored at -20°C overnight. DNA was pelleted by centrifugation, washed with 70 per cent ethanol, air-dried and resuspended in 30 µl nuclease-free water. RNase treatment was performed at 37°C and samples were stored at -20°C.

DNA quality was assessed by 0.8 per cent agarose gel electrophoresis with ethidium bromide staining, visualized under UV and quantified using a Nanodrop spectrophotometer. Dilutions (1:10) were prepared for further applications. The *mtCOI* gene was amplified using universal primers (LCO 1490 -5' GGTCAACAAATCATAAAGATATTGG 3' and HCO 2198 - 5' TAAACTTCAGGGTGACCAAA AAATCA 3') in 25 µl PCR reactions (Table 2). PCR products were confirmed on 0.8 per cent agarose gel under UV visualization. Sequencing was conducted at Eurofins Genomics India Pvt. Ltd. and sequences were analyzed using BLAST (NCBI) and deposited in GenBank.

Table 2

PCR thermal cycling condition

Step	Temperature (°C)	Duration (minutes)	Number of Cycles
Initial Denaturation	95	4	1
Denaturation	92	1	35
Annealing	54	1	35
Extension	72	1.5	35
Final Extension	72	10	1
Hold	4	Indefinite	-

Results and Discussion

Morphological Identification and Molecular Confirmation of Termite

Soldier (Table 3) : The head capsule yellow to reddish brown (Fig. 3). Antennae, labrum, pronotum and legs are pale yellowish brown; the abdomen is creamish white to pale yellow. The head is sparsely hairy and the body has a moderate amount of hair. The head capsule is subrectangular, with sides that are slightly straight and converge just before the antennae. The antennae consist of 17 segments, with segment 3 being much shorter than segment 2, segment 4 nearly matching the length of segment 2 and segment 5 being shorter than segment 4. The labrum is tongue-shaped with a bluntly rounded tip at the anterior end. The mandibles are robust and sabre-shaped, measuring 1.27-1.60 mm in length; the left mandible has a prominent tooth near the base of the middle third, while the right mandible has a small tooth slightly below the level of the left mandible's tooth. The postmentum is subrectangular, with sides bulging out in the proximal third. The pronotum is saddle-shaped, featuring a weak to moderately marked median notch on the anterior margin and a fairly distinct notch on the posterior margin.

Table 3

Morphometric data of the soldier caste of *Odontotermes horni*

Morphological characters	Measurements*
Head length to base of mandibles	2.47-3.00
Maximum head width	1.82-2.20
Left mandible length	1.27-1.60
Mandible head length index (left mandiblelength/head length to base of mandibles)	0.53-0.66
Tooth distance	0.70-1.00
Tooth distance to mandible length index	0.55-0.63
Length of postmentum	1.50-2.00
Maximum width of postmentum	0.75-0.93
Pronotum length	0.80-1.03
Pronotum width	1.40-1.80

*Measurements are in mm except indices

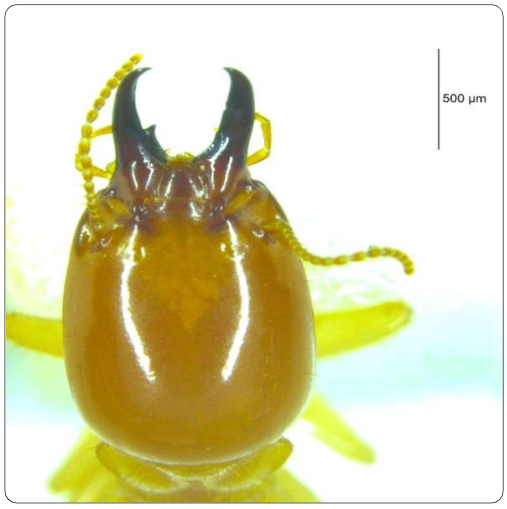

Fig. 3 : Dorsal view of the head of the soldier caste

Morphologically identified specimens were molecularly confirmed by comparing the similarity of obtained sequences with reference sequence using basic local alignment search tool (BLAST) at national centre for biotechnology information (NCBI) (Table 4). The *mtCOI* gene sequence with maximum coverage was deposited in GenBank and accession number PV034270 was obtained.

Isolation and Identification of Gut Bacteria

In this study, a total of 10 isolates were obtained (Table 5 & Table 6). 80 per cent of the isolated bacterial strains were identified as Gram-negative, while the remaining 20 per cent were Gram-positive.

TABLE 4

Molecular identification of *Odontotermes horni* based on COX1 gene sequence similarity using NCBI BLAST

Description	Scientific name	Percent identity	Accession No.
Odontotermes horni isolate S8 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Odontotermes horni*	97.23%	PQ285374
Odontotermes horni voucher NBAIR-T2 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Odontotermes horni*	96.19%	PQ146896.1

TABLE 5

Colony characterization of bacteria isolated from the gut of *Odontotermes horni*

Organism	Colour	Opacity	Margin	Shape	Elevation	Branching	Gram staining
Acinetobacter calcoaceticus	White to greyish-white	Opaque	Smooth	Circular	Raised	No	Negative
Acinetobacter johnsonii	White to greyish-white	Opaque	Smooth	Circular	Raised	No	Negative
Acinetobacter piscicola	White	Opaque	Smooth	Circular	Raised	No	Negative
Citrobacter freundii	Greyish	Opaque	Smooth	Circular	Raised	No	Negative
Proteus mirabilis	Colourless to pale	Translucent	Irregular	Irregular	Flat	No	Negative
Trabulsiella odontotermitis	Greyish-white	Opaque	Smooth	Circular	Raised	No	Negative
Escherichia fergusonii	Greyish-white to white	Opaque	Smooth	Circular	Raised	No	Negative
Escherichia hominis	Off white to white	Opaque	Smooth	Circular	Raised	No	Negative
Mammaliicoccus sciuri	White to cream	Opaque	Smooth	Circular	Raised	No	Positive
Staphylococcus gallinarum	White to cream	Opaque	Smooth	Circular	Raised	No	Positive

TABLE 6

List of bacterial species found in the gut of *Odontotermes horni*

Organism	Order	Family
Phylum - Pseudomonadota, Class - Gammaproteo bacteria		
Acinetobacter calcoaceticus	Pseudomonadales	Moraxellaceae
Acinetobacter johnsonii	Pseudomonadales	Moraxellaceae
Acinetobacter piscicola	Pseudomonadales	Moraxellaceae
Citrobacter freundii	Enterobacterales	Enterobacteriaceae
Proteus mirabilis	Enterobacterales	Enterobacteriaceae
Trabulsiella odontotermitis	Enterobacterales	Enterobacteriaceae
Escherichia fergusonii	Enterobacterales	Enterobacteriaceae
Escherichia hominis	Enterobacterales	Enterobacteriaceae
Phylum - Bacillota, Class - Bacilli		
Mammaliicoccus sciuri	Bacillales	Staphylococcaceae
Staphylococcus gallinarum	Bacillales	Staphylococcaceae

At the order level, the majority of the isolates belonged to Enterobacterales, comprising 50 per cent of the total bacterial population. This was followed by Pseudomonadales, which accounted for 30 per cent, while Bacillales represented the least dominant order, making up 20 per cent of the isolates (Fig. 4). These findings suggest that the gut of *O. horni* harbors a diverse microbial community with a significant prevalence of Gram-negative bacteria, particularly Enterobacterales and Pseudomonadales, which play critical roles in nutrient cycling and digestion within the termite gut ecosystem. This is consistent with previous reports on the gut microbiome of termites (Lo & Eggleton, 2011 and Culliney, 2013).

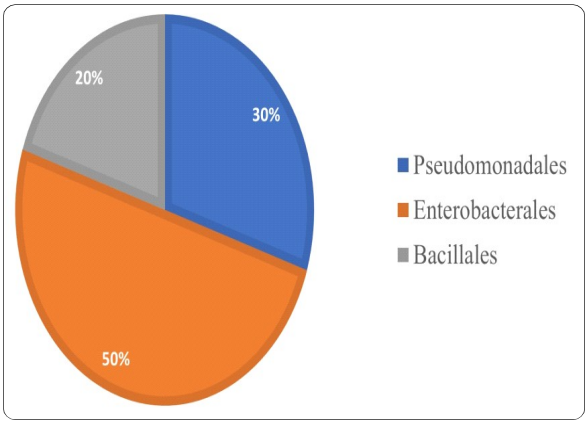

Fig. 4 : Relative abundance of different bacterial orders identified in the gut of *Odontotermes horni*

At the family level, Enterobacteriaceae was the most abundant, constituting 50 per cent of the isolates. Moraxellaceae was the second most prevalent family, contributing 30 per cent to the overall bacterial community, while Staphylococcaceae was the least represented family among the isolates (Fig. 5 and Table 6). Since the gut system is where ligno cellulosic material breakdown occurs, Enterobacteriaceae would be the predominant family in the majority of termite guts (Boucias *et al*., 2013). Members of this family have been widely reported to possess the capacity to degrade complex carbohydrates and play a key role in breaking down plant-based polymers into simpler compounds, which are then utilized by termites as a source of energy (Brune, 2014). These bacteria may also contribute to nitrogen fixation, an essential process that compensates for the nitrogen-poor diet of termites (Scharf, 2020).

Moraxellaceae, the second most prevalent family, likely aids in the degradation of lignin and cellulose, thereby facilitating the digestion of plant materials

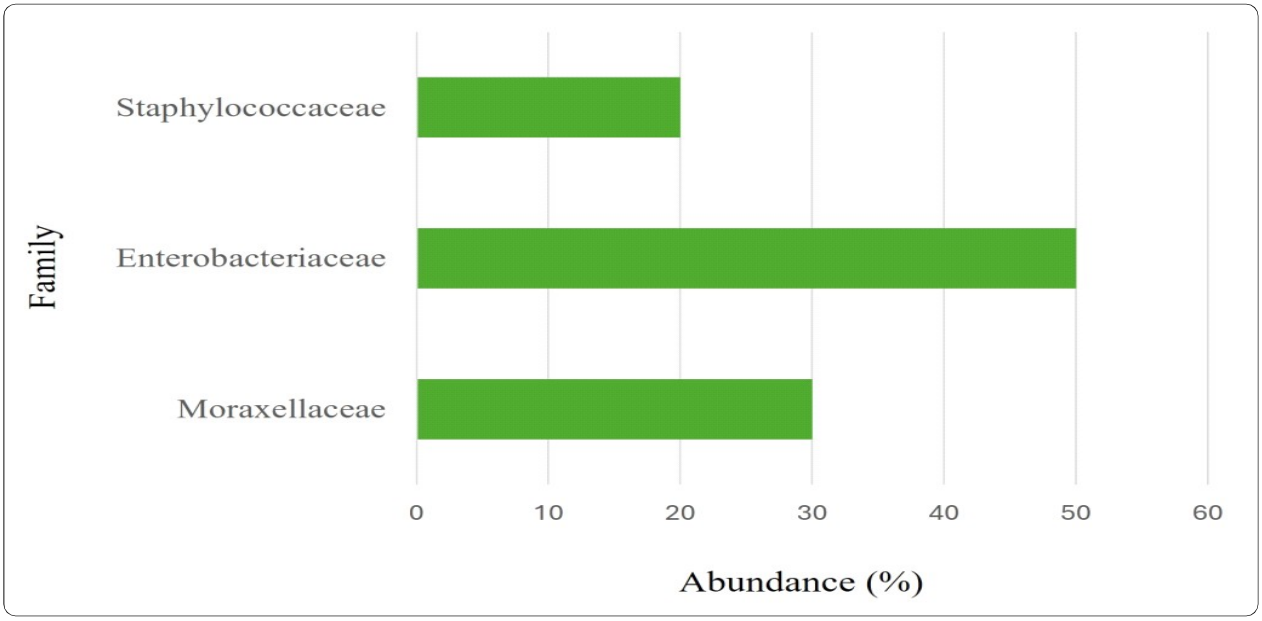

Fig. 5 : Distribution of bacterial families in the gut of *Odontotermes horni*

that termites consume (Engel and Moran, 2013). Members of this family have been observed in other insect gut microbiomes and are often involved in the detoxification of secondary plant metabolites, further enhancing their host's ability to efficiently utilize woody materials (Culliney, 2013). This symbiotic relationship underscores the ecological importance of gut bacteria in assisting termites in their role as ecosystem engineers, promoting the decomposition of plant matter and nutrient cycling in their environment (Waidele *et al*., 2019).

Despite being less abundant, the Gram-positive Bacillales and Staphylococcaceae may also play significant roles within the termite gut. Bacillales are well known for their ability to produce extra cellular enzymes capable of degrading polysaccharides and other complex carbohydrates, contributing to the digestion process (Brune, 2014). Furthermore, Bacillales are often associated with nitrogen fixation and cellulolytic activity, both of which are critical for nutrient cycling in termites (Jahnes & Sabree, 2020). Their presence indicates their likely contribution to the overall efficiency of the termite digestive system, especially in the breakdown of tough lignocellulosic biomass (Otani *et al*., 2014).

Staphylococcaceae, although not as commonly reported in termite gut, could be contributing to the maintenance of microbial diversity and gut homeostasis. They may have specialized roles in promoting gut health or protecting against pathogens, an area that warrants further investigation (Mudalungu *et al.,* 2021 and Lo & Eggleton, 2011). Their presence highlights the complex interactions between gut microbes, suggesting that even less dominant bacterial families play essential roles in maintaining the stability and functioning of the termite gut eco system (Engel *et al*., 2009).

Within the Enterobacterales order, *Citrobacter freundii*, *Proteus mirabilis*, *Trabulsiella odontotermitis*, *Escherichia fergusonii* and *Escherichia hominis* were isolated (Table 6). These bacteria are well-documented for their roles in breaking down lignocellulosic materials, particularly through the production of cellulases and hemicellulases, enzymes that degrade cellulose and hemicellulose into simpler sugars that termites can assimilate (Boucias *et al*., 2013; Brune, 2014 and Kavitha *et al.,* 2014).

Citrobacter freundii and *Proteus mirabilis* are known to contribute to nitrogen cycling within the termite gut by reducing nitrate to nitrogenous compounds, which offsets the termite's nitrogen-deficient diet (Scharf, 2020). The presence of *E. fergusonii* and *E. hominis*, both members of the Enterobacteriaceae family, further emphasizes the importance of these bacteria in maintaining the nutritional balance of the termite gut. These species likely contribute to nutrient absorption and vitamin synthesis, particularly by producing essential B-vitamins needed for termite metabolism. Such roles of gut microbiota in insects have been reviewed broadly by Engel and Moran (2013).

Trabulsiella odontotermitis, has been previously reported in the gut of termites and plays a similar role in lignocellulose degradation and nutrient recycling. The dominance of Enterobacteriaceae members highlights their critical role in supporting the termite's ability to digest tough plant fibers and recycle nitrogen, which is vital for the termite's survival in nutrient-poor environments (Boucias *et al*., 2013).

Within the Pseudomonadales order, *Acinetobacter calcoaceticus*, *Acinetobacter johnsonii* and *Acinetobacter piscicola* were isolated (Table 6). These bacteria are known for their versatile metabolic capabilities, including their ability to degrade complex organic materials and detoxify harmful compounds within the termite gut (Engel and Moran, 2013). The *Acinetobacter* species have also been associated with lignin degradation, which is crucial for the breakdown of woody materials consumed by termites. Moreover, these bacteria help detoxify secondary plant compounds, allowing termites to consume a wider range of plant materials without suffering from toxic effects (Culliney, 2013).

The Gram-positive isolates belonged to the Bacillales order, specifically the families Staphylococcaceae and Mammaliicoccaceae. *Mammaliicoccus sciuri* and *Staphylococcus gallinarum* were isolated, both of which are part of the gut microbiota involved in maintaining gut homeostasis and possibly providing defense against pathogens. These Gram-positive bacteria are less commonly associated with cellulose degradation but may play an auxiliary role in nutrient absorption and promoting microbial diversity (Jahnes and Sabree, 2020). The Staphylococcaceae family is also linked to the production of antimicrobial peptides, which may protect the termite gut from potential pathogens, ensuring a stable gut environment (Mudalungu *et al.,* 2021; Warnecke *et al*., 2007 and Lo & Eggleton, 2011).

Although Gram-positive bacteria are less abundant, their presence in the termite gut suggests specialized roles that complement the activities of Gram-negative bacteria. For instance, *M. sciuri* has been associated with biofilm formation, which could enhance microbial colonization and stability within the termite gut, while *S. gallinarum* may contribute to maintaining a balanced microbial ecosystem by suppressing harmful bacterial overgrowth (Kavitha *et al*., 2014 and Otani *et al*., 2014).

The functional diversity of the termite gut microbiota, with bacteria contributing to lignocellulose degradation, nitrogen fixation and defense against pathogens, highlights the evolutionary success of termites as ecosystem engineers. Their ability to break down plant biomass and recycle nutrients plays a crucial role in maintaining soil fertility, emphasizing the ecological importance of these microorganisms not only for termite survival but also for broader ecosystem health (Culliney, 2013; Waidele *et al*., 2019 and Wu & Chou, 2009).

These findings highlight the intricate microbial community residing within the gut of *Odontotermes horni*, with a marked dominance of Gram-negative bacteria, particularly members of the Enterobacteriaceae and Moraxellaceae. These bacteria play pivotal roles in lignocellulose degradation, nitrogen fixation and detoxification-functions essential for the termite's adaptation to a nutrient-poor, plant-based diet. Though less abundant, Gram-positive bacteria such as *Mammaliicoccus sciuri* and *Staphylococcus gallinarum* may contribute to gut homeostasis and defense through antimicrobial activity and biofilm formation.

The potential functional diversity of these gut microbes may contribute to termite survival and suggests possible roles in organic matter decomposition and soil fertility. While the current study does not directly assess the metabolic functions of these bacterial groups, previous research highlights their involvement in key processes such as nitrogen fixation and cellulase production. Future investigations using functional genomics or enzymatic assays would be valuable to elucidate the specific metabolic pathways employed by these microbes and to better understand their symbiotic relationships with termites and their environmental significance.

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