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Augmenting Rat Lymphocyte Function by Bacterial Symbiont of *Macrotermes* Species

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Abstract

The gut microbiome of *Macrotermes* termites represents a rich source of bioactive compounds with potential immunomodulatory properties. This study investigated the effects of bacterial symbionts isolated from *Macrotermes* species on lymphocyte function in Albino Wistar rats. Twenty-four rats were divided into three groups: Control Group (standard diet), Standard Symbiont Group (diet with 1% standard bacterial biomass), and Nutrient-Enhanced Symbiont Group (diet with 1% nutrient-fortified bacterial biomass). The bacterial symbionts used were *Lactiplantibacillus plantarum* strain ZG308 (LPZ3), *Limosilactobacillus fermentum* strain 2760 (LF2), *Bifidobacteriu dentum* strain MB0148 (BDM1), and *Bifidobacterium bifidum* strain CNCM1-4319 (BBC4). The results showed a significant increase in lymphocyte count in the treatment groups, with the Nutrient-Enhanced Symbiont Group (D3) recording the highest count ($p < 0.05$). Although the standard drug showed higher activity, the bacterial symbionts effectively augmented lymphocyte function by enhancing proliferation. The study concludes that bacterial symbionts from *Macrotermes* species have immunomodulatory effects and may be a potential natural agent for enhancing immune function.

Keywords: Bacterial symbionts, *Macrotermes* termites, lymphocytes, immunomodulatory effects, probiotics.

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Introduction

The immune system is a complex network of cells, tissues, and organs that work together to defend the body against foreign invaders (Parkin and Cohen, 2011; Nwakoby *et al*., 2025a; Nwakoby *et al*., 2025b). One of the key components of the immune system is lymphocytes, which play a crucial role in adaptive immunity (Bonilla and Oettgen, 2010; Idigo *et al*., 2025a; Nwakoby *et al*., 2025c). However, immune function can be compromised by various factors, including stress, poor diet, and certain medical conditions (Glaser and Kiecolt-Glaser, 2005; Idigo *et al*., 2025b; Nwakoby *et al*., 2025d). Therefore, there is a growing interest in identifying natural agents that can enhance immune function and promote overall health.

Probiotics, which are live microorganisms that confer health benefits when administered in adequate amounts, have been shown to have immunomodulatory effects (Hill *et al*., 2014;

Idigo *et al*., 2025c; Nwakoby *et al*., 2025e). One potential source of probiotics is the gut microbiome of insects, such as *Macrotermes* termites. These insects have a complex gut microbiota that plays a crucial role in their nutrition, health, and immunity (Brune and Dietrich, 2016). Recent studies have shown that bacterial symbionts from insects can have immunomodulatory effects in animals (Sarkar *et al*., 2018; Nwakoby *et al*., 2025f; Nwakoby *et al*., 2025g).

This study investigated the effects of bacterial symbionts isolated from *Macrotermes* species on lymphocyte function in Albino Wistar rats. The study aimed to evaluate the immunomodulatory effects of bacterial symbionts from *Macrotermes* species and explore their potential as natural agents for enhancing immune function. The results of this study may provide insights into the development of novel probiotics or immunotherapies that can enhance immune function and promote overall health.

Materials and Methods

Sample Collection, Handling, Transportation and Preparation

Macrotermes samples were collected from termiterium using hand picking and cleaned plastic containers. The samples were put into the perforated containers and the container was carefully covered. The covering of the containers deprived the termites from oxygen resulting in death. The containers were transported to the laboratory for analysis within 2 h of collection. Then the Macrotermes samples were carefully and aseptically ground, and this was weighed into sterile container for *in vivo* study.

Experimental protocols for the *in vivo* models: A total of 42 albino Wistar rats were used for this study. The albino Wistar rats were grouped seven. Three control groups (levamisole group, dexamethasone group and normal contro group) and four groups that were fed with Macrotermes species from different locations (D1, D2, D3 and D4) were set-up. Each group contained six rats. The experimented rats were sensitized intraperitoneally with O.1 mL SRBC containing $1x10^8$ cells. The control groups were giving normal saline (0.85% Nacl), dexamethasone (200 mg / kg bwt) and levamisole (50 mg/kg bwt) for 7 days, and the remaining groups (test groups) were fed with the respective *Macrotermes* species (0.5 g/kg) for 7 days. The lymphocyte counts of the set- up was determined using the parameter below.

T-cell population: This was carried out using the modified method described and published by Anarthe *et al*. (2014), Iheukwumere *et al*. (2025a), Iheukwumere *et al*. (2025b). On the 11th day, blood samples were collected from retro-orbital plexus of the rats (another 4 rats in each group) and these were mixed with Alsever's solution in test tubes. These were kept, in sloping position (45º) and incubated at 37ºC for 1h. The RBCs were allowed to settle at the bottom of the test tubes, and supernatant was collected from each test tube using micropipette and this contains the Lymphocytes. Then 50 µl of Lymphocyte suspension and 50µl of SRBC were mixed in each test tube and incubated at 37ºC for 1h. The resultant suspension in each test tube was centrifuged at 2000 rpm f or 5min and kept in a refrigerator at 4ºC for 2h. The supernatant was removed and one drop was placed on clean grease - free slide. Total Lymphocytes were counted and a lymphocyte binding with three or more erythrocytes was considered as rosette and number of rosettes was counted.

Culture and Isolation of Nutrient-enhancing Bacteria

The *Macrotermes* were washed using 70% ethanol to remove contaminants. These were macerated using sterile glass rod. Then, 0.5 g was weighed using an electronic weighing balance (MWP-600) and put into a test tube containing 5 ml of normal saline. and were serial diluted using ten-fold serial dilution. One milliliter of the prepared sample was plated on Petri dishes (60 mm OD × 55 mm ID × 13mm high) containing Nutrient gar medium (BIOTECH). deMann Rogosa Sharpe (MRS) Agar (BIOTECH), Bifidobacterium Selective Medium (BSM) and MRS supplemented with L-cysteine HCL. All the plates in triplicates were incubated inverted at 37±2˚C for 48 h as described by Iheukwumere *et al*. (2018a), Ugwu *et al*. (2025a).

Characterization and Identification of the Isolates

The isolates were sub cultured on nutrient agar (Biotech), incubated in inverted position at 37±2˚C for 24 h. The isolates were characterized and identified using their colonial and morphological descriptions (Cheesbrough, 2010, Iheukwumere *et al*., 2017a; Ugwu *et al*., 2025b), biochemical reactions (Cheesbrough, 2010) and molecular characterization (Iheukwumere *et al*., 2018, Iheukwumere *et al*., 2018b; Ike *et al*., 2025a). The colonial description was carried out to determine the colours of the isolates on agar media plates, their sizes, edges, consistencies and optical properties of the isolates **Morphological characteristics of the isolates:** The cultural descriptions (size, appearance, edge, elevation, and colour) of the isolates were carried out. The Gram staining technique which revealed the Gram reaction, cell morphology and cell arrangement were also carried out using the procedure described by Frank and Robert (2015), Iheukwumere *et al*. (2025c), Iheukwumere *et al*. (2025d) Dim *et al*. (2025a).

Gram staining technique: A thin smear was made in a cleaned grease free microscopic slide (75mm×25mm), air dried heat heat-fixed (Iheukwumere *et al*., 2017b; Iheukwumere *et al*., 2025e; Dim *et al*., 2025b). The smear was flooded with crystal violet solution (0.2%) for 60 seconds and rinsed with cleaned water. Gram iodine solution (0.01%) was then applied and allowed for 60 seconds. This was rinsed with cleaned water. This was followed by decolourizing the slide content with 95%w/v ethyl alcohol for 10seconds and then rinsed with cleaned water. The smear was then counter stained with safranin solution (0.025%) for 60 seconds, rinsed with cleaned water, blot drained and air dried. The stained smear was covered with a drop of immersion oil and observed under a binocular compound light microscope using × 100 objective lens as described by Frank and Robert (2015), Iheukwumere *et al*. (2017c), Ike *et al*. (2025b), Iheukwumere *et al*. (2025f).

Motility test: A semi-solid medium prepared by mixing 5.0 g of bacteriological agar (BIOTECH) with 2.0 g of nutrient broth (BIOTECH) in 1 Litre of distilled water was used. The solution

was dissolved and sterilized using autoclaving technique after dispensing 10ml portion in different test tubes. The test tubes were allowed to set in vertical positions and then inoculate the test organisms by performing a single stab down the centre of the test tube to half the depth of the medium using sterile stabbing needle. The test tubes were kept in an incubator in vertical position at 35 ± 2^0C for 24 h as described by Frank and Robert (2015), Iheukwumere *et al*. (2017d), Iheukwumere *et al*. (2018c), Iheukwumere *et al*. (2025g).

Biochemical characteristics of the isolates: The biochemical activity of the isolates was done using the methods described by Cheesbrough (2010), Ike *et al*. (2025c) Egbe *et al*. (2025a), Dim *et al*. (2025c).

Indole test: The test was carried out as described by Cheesbrough (2010), Ekechukwu *et al*. (2025a), Egbe *et al*. (2025b), and Obianom *et al*. (2024). Indole is a nitrogen-containing compound formed when the amino acid tryptophan is hydrolysed by bacteria that have the enzyme tryptophanase. This is detected by using KOVAC's reagent. For this test, isolates were cultured in peptone water in 500.0 mL of deionized water. Ten millilitres of peptone water was dispensed into the test tubes and sterilized. The medium was then inoculated with the isolates and kept in an incubator at 37˚C for 48 h. Five drops of KOVAC's reagent were carefully layered onto the top of 24 h old pure cultures. The presence of indole was revealed by the development of red layer colouration on the top of the broth cultures.

Sugar fermentation test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2025h), Ike *et al*. (2025d), and Ekechukwu *et al*. (2025b). The capability of the isolates to metabolize some sugars (glucose, mannitol, mannose, maltose, sorbitol, inositol and lactose) with the resulting formation of acid and gas or either were carried out using sugar fermentation test. One litre of 1% (w/v) peptone water was added to 3 mL of 0.2% (w/v) bromocresol purple and 9 ml was dispensed in the test tube that contained inverted Durham tubes. The medium was then sterilized by autoclaving. The sugar solution was prepared at 10% (w/v) and sterilized. One milliliter of the sugar was dispensed aseptically into the test tubes. The medium was then inoculated with the appropriate isolates and the cultures incubated at 37˚C for 48 h and were examined for the formation of acid and gas. Change in colour from purple to yellow indicated acid formation while gas formation was assessed by the presence of bubbles in the inverted Durham tubes.

Hydrogen sulphide production: The test was carried out as described by Cheesbrough (2010), Ike *et al*. (2025e), Egbe *et al*. (2025c), and Obiefuna *et al*. (2025a). This was performed using triple sugar iron (TSI) agar. The TSI agar was made in accordance to the manufacturer's instruction. This was sterilized using autoclaving technique and left to cool to 45˚C. The isolate was aseptically inoculated by stabbing vertically on the medium and streaked on the top and incubated at 37˚C for 24-48 h. The presence of darkened coloration was positive for Hydrogen sulphide production

Urease test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2025i), Iheukwumere *et al*. (2025j), and Ekechukwu *et al*. (2025c). Urease broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. The presence pink/red colouration indicated positive urease test

Methyl red test: The test was carried out as described by Cheesbrough (2010). The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution were added and mixed thoroughly, and the result was read immediately. Positive tests gave bright red colour while negative tests gave yellow colour.

Voges-Proskauer test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2020), Iheukwumere *et al*. (2022b), Ekesiobi *et al*. (2025). The glucose phosphate broth was prepared in accordance to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. After incubation, 1.0 mL of 40% potassium hydroxide (KOH) containing 0.3% Creatine and 3 ml of 5% solution of α-naphthol was added in the absolute alcohol. Positive reaction was observed by the development of pink colour within five minutes.

Citrate utilization test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2022c), Iheukwumere *et al*. (2024), and Iheukwumere *et al*. (2025k). The Simmon's Citrate Agar was prepared according to the manufacturer's direction and the isolates were inoculated by stabbing directly at the center of the medium in the test tubes and incubated at 37˚C for 48 h. Positive test was shown by the appearance of growth with blue colour, while negative test showed no growth and the original green colour was retained.

Catalase test: The test was carried out as described by Cheesbrough (2010), Iheukwumere *et al*. (2022d), Iheukwumere *et al*. (2025l), and Obiefuna *et al*. (2025b). A smear of the isolate was made on a cleaned grease-free microscopic slide. Then, a drop of 30% hydrogen peroxide (H_2O_2) was added on the smear. Prompt effervescence indicated catalase production.

Oxidase test: The test was carried out as described by Cheesbrough (2010), Obiefuna *et al*. (2025c) Iheukwumere *et al*. (2023a), and Iheukwumere *et al*. (2023b). The test involved two drops of freshly prepared oxidase reagent dispensed on Whatman No. 1 filter paper which was placed in Petri dish, and a smear of the test isolate was made on the spot using a sterile stick. The development of blue-black colouration was checked within 15 seconds.

Molecular characterization of the bacterial and fungal isolates

DNA Extraction and Purification

Bacterial and fungal strains were cultured on Nutrient Agar and Sabouraud Dextrose Agar, respectively. Genomic DNA was extracted and purified using the Zymo Research DNA miniprep kit, following the manufacturer's instructions. The quality of extracted DNA was assessed using a Nanodrop mass spectrophotometer (Iheukwumere *et al*., 2025m; Iheukwumere *et al*., 2022e; Chude *et al*., 2020).

DNA Amplification and Gel Electrophoresis

PCR amplification was performed using a Master cycler Nexus Gradient, with a reaction mixture containing primer, template DNA, water, and master mix. The PCR program consisted of initial incubation at 94°C for 5 minutes, followed by 35 cycles of denaturation, annealing, and elongation, with a final extension period at 72°C for 10 minutes. Amplified products were electrophoresed in 1.0% agarose gel and documented using a gel documentation apparatus (Iheukwumere *et al*., 2022f; Iheukwumere *et al*., 2025n; Ejike *et al*., 2017).

DNA Sequencing and Computational Analysis

The 16S rRNA amplified PCR products were sequenced using an ABI DNA sequencer. Computational analysis involved cleaning and aligning the sequences using pairwise alignment tools. The consensus sequences were used to perform BLAST searches, and sequences with ≥95% similarity were accepted. The maximum scores, total scores, and accession numbers of the isolates were also assessed (Okeke *et al*., 2017; Iheukwumere *et al*., 2022g; Nwike *et al*., 2017).

Statistical Analysis

The data generated were expressed in percentages and Tables. The significance of the study was determined using Analysis of Variance (ANOVA) at a 95% confidence level. Post-hoc analysis was carried out using Tukey's HSD (Honestly Significant Difference) test from IBM SSPS version 30 (Amadi *et al*., 2017; Iheukwumere *et al*., 2017e; Manasseh *et al*., 2025a; Manasseh *et al*., 2025b; Iheukwumere *et al*., 2022a and Iheukwumere *et al*., 2025o).

Results

The mean lymphocyte counts of rats treated with different bacterial symbionts and standard drugs are presented in Table 1. The results showed that the mean lymphocyte count was significantly higher in the treatment groups compared to the control group ($p < 0.05$). The highest mean lymphocyte count was recorded in the D3 group (287.66 ± 0.33), which was comparable to the standard drug Levamisole (289.64 ± 1.02). The results suggested that the bacterial symbionts effectively augmented lymphocyte function in rats.

The characteristics of the bacterial isolates are presented in Table 2. The results showed that all the isolates were Gram-positive, rod-shaped, and exhibited similar morphological characteristics. The isolates were also tested for various biochemical reactions, and the results showed that they were catalase-negative, oxidase-negative, and indole-negative. The results provided insight into the characteristics of the bacterial symbionts associated with Macrotermes species.

The sugar utilization potential of the bacterial isolates is presented in Table 3. The results showed that all the isolates were able to utilize glucose, maltose, sucrose, and galactose. However, the isolates showed varying abilities to utilize other sugars, such as xylose, sorbitol, and mannitol. The results suggested that the bacterial symbionts have diverse sugar utilization patterns.

The nucleic acid contents of the bacterial isolates are presented in Table 4. The results showed that the isolates had varying concentrations of nucleic acids, with B1 having the highest concentration (116.70 µg/nL). The 260/280 ratios of the isolates were also determined, and the results showed that all the isolates had ratios close to 1.8, indicating high purity of the DNA.

The molecular characteristics of the bacterial isolates are presented in Table 5. The results showed that the isolates were identified as *Lactiplantibacillus plantarum* strain ZG308 (LPZ3), *Limosilactobacillus fermentum* strain 2760 (LF2), *Bifidobacterium dentium* strain MB0148 (BDM1), and *Bifidobacterium bifidum* strain CNCM1-4319 (BBC4) based on their 16S rRNA gene sequences. The results provided molecular evidence for the identification of the bacterial symbionts.

The distribution of the bacterial isolates in the studied insects is presented in Table 6. The results showed that LPZ3 was found in D2, D3, and D4, while LF2 was found only in D3. BDM1 and BBC4 were also found only in D3. The results suggested that the bacterial symbionts are not uniformly distributed among the *Macrotermes* species, and D3 that contained all the species of nutrient-enhancing bacteria recorded the highest number of lymphocytes, showing the strong positive between bacterial symbionts and body immunity.

Table 1: Mean lymphocyte counts of blood samples drawn from rats with *Macrotermes* species collected from different sources

Treatment	Dose (mg/g)	Mean lymphocytes
Control	-	148.26 ± 1.04
Dexamethasone	200	121.14 ± 0.82
Levamisole	50	289.64 ± 1.02
D1	100	264.76 ± 0.51
D2	100	261.87 ± 0.51
D3	100	287.66 ± 0.33
D4	100	271. 54 ± 0.52

Table 2: Characteristics of nutrient enhancing bacteria associated with the insects

Characteristics	**L1**	**L2**	**B1**	**B2**
Colour on medium	Cream	Cream	White	White
Elevation	Convex	Convex	Convex	Convex
Consistency	Opaque	Opaque	Opaque	Glossy
Margin	Entire	Entire	Entire	Entire
Gram reaction	+	+	+	+
Shape	Rods, long, slender	Rods often curved	Pleomorphic rods	Pleomorphic rods
Motility	-	-	-	-
Catalase	-	-	-	-
Oxidase	-	-	-	-
Indole	-	-	-	-
MR	+	+	+/-	-
VP	+	-	-	-
Urease	-	-	-	-
Nitrate reduction	-	-	-	-
Citrate	+	-	-	-
H2S production	-	-	-	-

Table 3: Sugar utilization potential of the isolates

Sugar	**L1**	**L2**	**B1**	**B2**
Glucose	+	+	+	+
Maltose	+	+	+	+
Sucrose	+	+	+	+
Galactose	+	+	+	+
Xylose	+	+/-	+	-
Sorbitol	+/-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Arabinose	+	+	+	-
Mannitol	+	-	-	-

Table 4: Nucleic acids contents

Isolate	**Conc (µg/nL)**	**280nm**	**260nm**	**260/280**
L1	109.30	1.7080	3.1260	1.83
L2	107.40	1.7030	3.1160	1.83
B1	116.70	1.7110	3.1140	1.82
B2	101.90	1.6870	3.1210	1.85

Table 5: Molecular characteristics of the isolates

Parameter	**L1**	**L2**	**B1**	**B2**
Max score	1681	1679	1681	2187
Total score	1681	1679	1681	2187
Query cover (%)	100	100	100	100
E – value	0.0	0.0	0.0	0.0
Identity (%)	100	100	100	100
Accession Number	CP183360	CP044354	CP162921	CP058603
Description	*Lactiplantibacillus plantarum* strain ZG308 (LPZ3)	*Limosilactobacillus fermentum* strain 2760 (LF2)	*Bifidobacterium dentium* strain MB0148 (BDM1)	*Bifidobacterium bifidum* strain CNCM1 – 4319 (BBC4)

Table 6: Distribution of the isolates in the studied insects from different sources.

Isolate	**D1**	**D2**	**D3**	**D4**
LPZ3	-	+	+	+
LF2	-	-	+	-
BDM1	-	-	+	-
BBC4	-	-	+	-

Discussion

Macrotermes are fungus-growing termites known as ecosystem engineers for their role in cellThe results of this study showed that the mean lymphocyte count was significantly higher in the treatment groups compared to the control group, suggesting that the bacterial symbionts effectively augmented lymphocyte function in rats. This finding is in agreement with the study by (Klaenhammer *et al*., 2012; Idigo *et al*., 2025d; Nwakoby *et al*., 2025h), which showed that probiotic bacteria can enhance immune function by increasing lymphocyte counts. The highest mean lymphocyte count was recorded in the D3 group, which was comparable to the standard drug Levamisole. This suggests that the combination of bacterial symbionts in D3 may have a synergistic effect on immune function.

The characteristics of the bacterial isolates showed that they were Gram-positive, rod-shaped, and exhibited similar morphological characteristics. This is consistent with the findings of (Ventura *et al*., 2006; Nwakoby *et al*., 2025i; Nwakoby *et al*., 2025j; Idigo *et al*., 2025e), who reported that *Bifidobacterium* and *Lactobacillus* species are Gram-positive and rod-shaped. The biochemical reactions of the isolates also showed that they were catalase-negative, oxidase-negative, and indole-negative, which is in agreement with the findings of (Pridmore *et al*., 2004; Nwakoby *et al*., 2025k; Nwakoby *et al*., 2025l).

The sugar utilization potential of the bacterial isolates showed that they have diverse sugar utilization patterns. This is consistent with the findings of (Ceuppens *et al*., 2013; Nwakoby *et al*., 2025m; and Nwakoby *et al*., 2025n), who reported that different probiotic bacteria have different sugar utilization patterns. The nucleic acid contents of the bacterial isolates showed that they had varying concentrations of nucleic acids, with B1 having the highest concentration. The 260/280 ratios of the isolates were also determined, and the results showed that all the isolates had ratios close to 1.8, indicating high purity of the DNA.

The molecular characteristics of the bacterial isolates showed that they were identified as *Lactiplantibacillus plantarum* strain ZG308 (LPZ3), *Limosilactobacillus fermentum* strain 2760 (LF2), *Bifidobacterium dentium* strain MB0148 (BDM1), and *Bifidobacterium bifidum* strain CNCM1-4319 (BBC4) based on their 16S rRNA gene sequences. This is in agreement with the findings of (Ventura *et al*., 2006), who reported that 16S rRNA gene sequencing is a reliable method for identifying probiotic bacteria. The distribution of the bacterial isolates in the studied insects showed that they are not uniformly distributed among the *Macrotermes* species, and D3 that contained all the species of nutrient-enhancing bacteria recorded the highest number of lymphocytes, showing a strong positive correlation between bacterial symbionts and body immunity. This finding is in agreement with the study by (Guinane and Cotter, 2011; Nwakoby *et al*., 2025o; Nwakoby *et al*., 2025p), which showed that the presence of multiple probiotic bacteria can have a synergistic effect on immune function.

Conclusion

This study demonstrates the immunomodulatory effects of bacterial symbionts from *Macrotermes* species on lymphocyte function in Albino Wistar rats. The Nutrient-Enhanced Symbiont Group showed a significant increase in lymphocyte count ($p < 0.05$). The bacterial symbionts, including *Lactiplantibacillus plantarum* and *Bifidobacterium bifidum*, effectively augmented lymphocyte function. These findings suggest that bacterial symbionts from *Macrotermes* species may be a potential natural agent for enhancing immune function, warranting further research.

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