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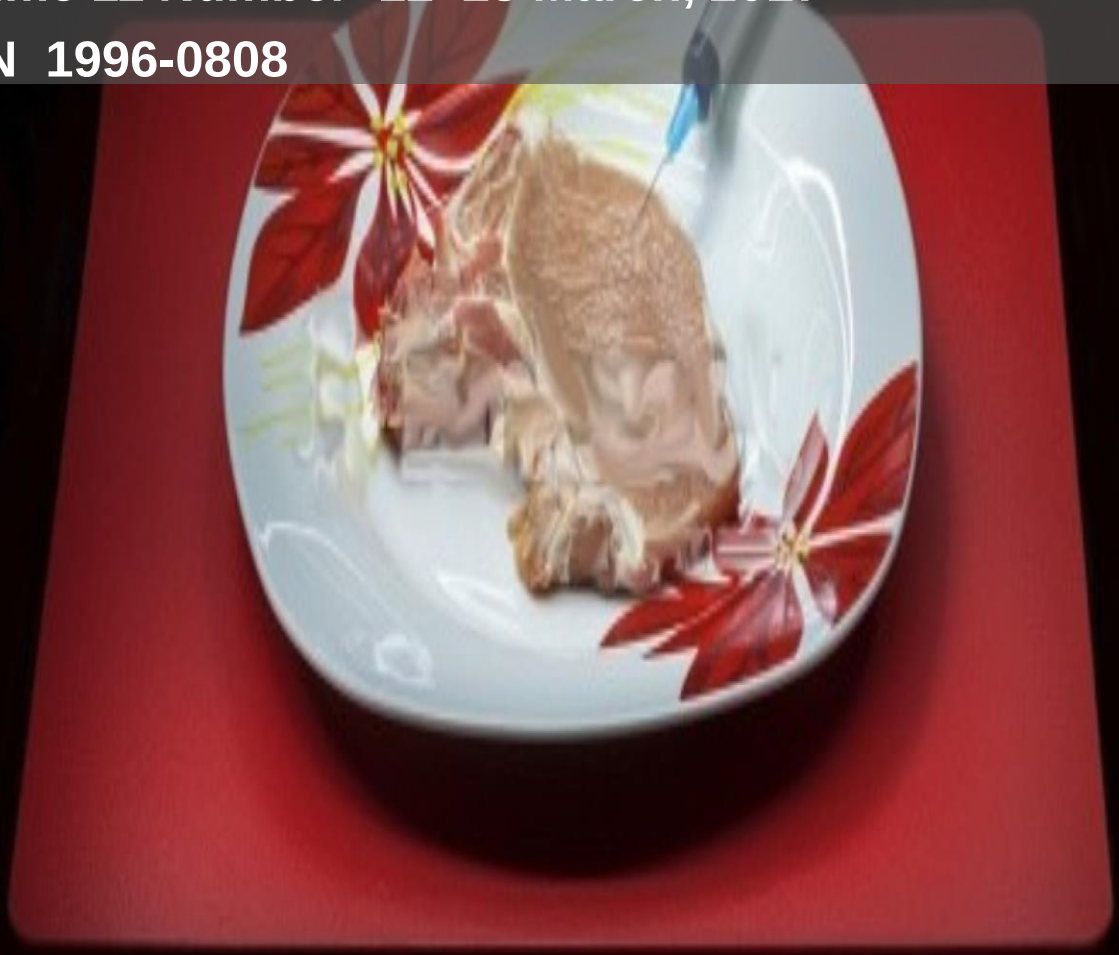

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Full Length Research Paper

Isolation and characterization of potential probiotic enterococci strains from soft cheese flora

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This study was conducted to determine the *in-vitro* probiotic properties of *Enterococcus faecium* strains isolated from soft cheese. To evaluate the safety of *Enterococcus* strains, we compared the pathogenic genes, antimicrobial susceptibility of the probiotic strains to those of clinical isolates, and their antimicrobial activity against food-borne pathogenic and spoilage bacteria. *Enterococcus* strains were identified and evaluated *in vitro* for biochemistry methods acid, bile salts, lysozyme and pancreatin tolerance. One hundred and three strains were identified as *E. faecium*, and none of them were no vancomycin-resistant, and no pathogenic genes – such as *cyl*A, *asa*1, *gel*E, *ace* and *cpd* – were found. The isolates showed good viability at 120 and 240 min of incubation with pH 3.0, and were able to resist 0.3% and 0.1 g/ml of bile salts and pancreatic enzyme, respectively. One observed strong autoaggregation phenotype, and the isolates demonstrated high activity against *L. innocua, L. monocytogenes, E. faecalis S. aureus*, *Salmonella* Enteritidis and *Salmonella* Typhimurium. The results instigate the continuity of studies of *E. faecium* isolates in order to obtain a known probiotic strain.

Key words: *Enterococcus,* good bacteria, pathogenic genes, foods, antimicrobial activity.

INTRODUCTION

The use of *Enterococcus* spp. in the making of fermented foods, such as milk, yogurt, cheese, fermented sausages and vegetables (Foulquié Moreno et al., 2006) has a long record in the history of food. Selected *Enterococcus* strains have been employed as probiotics in the promotion of both human and animal health ,improving the intestinal microbial balance (Foulquié Moreno et al., 2006; Franz et al., 2011) and producing enterocins (antimicrobial peptides) to inhibit the growth of food-borne pathogenic and spoilage bacteria (Ogaki et al., 2016).

Other therapeutic or prophylactic properties associated with probiotic enterococci include the improvement of constipation and diarrhea, reduction in cholesterol levels, stimulation of immunity and suppression of the carcinogenesis (Agerholm-Larsen et al., 2000; de Roos and Katan, 2000; Parvez et al., 2006; Meurman and Stamatova, 2007; Candela et al., 2008).

However, presence of enterococci in foods may present conflicting effects, either as a risk, a foreign (?) or as an indicator of poor hygiene during the processing of

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food (Bhardwaj et al., 2008). Some types of *Enterococcus* produce virulence factors (Jett et al., 1994; Foulquié Moreno et al., 2006), and are sometimes associated with pathogenicity (Khan et al., 2010). They have been reported to be the cause of endocarditis, bacteraemia, and several infections, besides multiple antibiotic resistances (Kayser, 2003). In addition, vancomycin-resistant *Enterococcus* (VRE) emerged and has become a major public health problem in several countries (Foulquié Moreno et al., 2006).

One cannot presume whether a specific probiotic bacterium will have a beneficial effect on health, except through determination of its genus or species. Reports on the safety of probiotics are limited, and there are few details about the nature of probiotic bacterial species (Sanders et al., 2010; Fijan, 2014). As part of the selection of new probiotic enterococci candidates, one needs to do a series of *in vitro* and *in vivo* analyses to assess their probiotic properties. Carrying no virulence factors nor vancomycin-resistant genes is a prior condition to regard an enterococci candidate as safe and eligible to be used as a starter of cultures, co-cultures; on the other hand, the probiotics that are acceptable for the preparation of food and medicines for humans are those which occur naturally in the intestinal tract of healthy human subjects and foods (Sanders et al., 2010).

Other criteria for potential probiotic strains should include their ability to colonize the intestinal tracts of humans and other mammals (Verschuere et al., 2000), and their resistance to survive humans' biological barriers, such as the strains that have proven ability to survive the gastrointestinal tract (Dunne et al., 2001; Vinderola and Reinheimer, 2003), the presence of proteolytic enzymes and low pH values, bile salts and pancreatic juices.

Probiotic cultures should also be antagonistic to pathogenic bacteria by producing antimicrobial substances and must be safe for human use, maintaining their viability and beneficial properties during manufacturing processes (Schillinger et al., 2005).

Therefore, the objective of this investigation was to perform a characterization of new food enterococcal strains of cheese origin and elicit their potential application as probiotics.

MATERIALS AND METHODS

Bacterial strains and culture preparation

The study comprised one hundred and three *Enterococcus* spp. strains isolated from artisanal soft cheeses. Such isolates were identified as members of the *Enterococcus* spp. based on the phenotypic and genotypic criteria as previously reported (Furlaneto-Maia et al., 2014). A single probiotic culture containing strain *Lactobacillus acidophilus* LA-5 was used as control (Chr. Hansen). The bacterial strains were reactivated in MRS (Merck, Darmstadt, Germany) broth medium for 18 h at 37ºC under shaking conditions. Cells were harvested by centrifugation at 10000 g for 5 min and washed twice in NaCl solution (0.85% w/v). The pellet was resuspended in physiologic solution in order to obtain a suspension that contained approximately 10^9-10^{10} CFU/mL.

Antimicrobial susceptibility testing

Antibiotic discs (Laborclin®) were used to determine the strains susceptibility to ampicillin (AMP, 10 µg), nalidixic acid (NAL, 30 µg), vancomycin (VAN, 30 µg), erythromycin (ERY, 15 µg), chloramphenicol (CLO, 30 µg), norfloxacin (NOR, 10 µg), tetracycline (TET, 30 µg), imipenem (IPM 10 µg), amikacin (AK, 30 µg); cephalothin (CF, 30 µg); ciprofloxacin (CIP 5 µg); amoxicillin/clavulanic acid (AMC, 30 µg). The discs were placed onto Mueller–Hinton agar plates overlayed with the enterococcal culture with cell concentration corresponding to 0.5 McFarland standard turbidity. After incubation at 37°C for 18-24 h, the diameter of inhibition haloes around the colonies was measured. Susceptibility or resistance was interpreted in accordance with the Clinical Laboratory Standard Institute (CLSI, 2011) recommendations, and *Staphylococcus aureus* 25923 ATCC were used as strain quality control.

Determination of virulence factors

Enterococcus spp. genomic DNA was extracted by boiling method (Furlaneto-Maia et al., 2014). Determination of virulence factors was performed using a polymerase chain reaction (PCR) method. PCR assay was carried out using species-specific primers (Table 1). All PCR amplifications were performed in a final volume of 20 µl containing 1 ρmol of each primer (Forward e Reverse), 0.17 mM dNTPs, 2.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (Invitrogen), buffer of Taq, and 10 µl template DNA. One observed an initial cycle of denaturation (94°C for 2 min), followed by 30 cycles of denaturation (94°C for 1 min), annealing at an appropriate temperature (Table 1) for 1 min and elongation (72°C for 10 min). A thermal cycler (Techne-Tc3000) was used to perform the PCR reactions. PCR products were analysed by gel electrophoresis in 1.5% agarose stained with ethidium bromide (0.5 $g.ml^{-1}$), observed under UV transillumination and photographed with L-PIX ST (LOCCUS).

Hemolytic activity

To investigate the production of hemolysin, the strains grown in MRS broth were streaked onto layered agar plates with 7% v/v fresh sheep blood (Himedia), then grown at 37°C for 48 h. β-hemolysis was revealed by the formation of clear zones surrounding the colonies on the blood agar plates (Foulquié Moreno et al., 2006).

Effects of low pH on growth rate

The effects of low pH on growth rate were determined as previously described by Oluwajoba et al. (2013), with modifications. *Enterococcus* spp. bacterial colonies were incubated for 0, 1, 2, 3 and 4 h at 37ºC in MRS medium, then adjusted to pH 3 with HCl (4 mol/l). The number of CFU/ml was calculated and compared to the CFU/ml at time 0. The surviving bacteria were counted on the MRS agar, and all these experiments were performed in triplicate.

Lysozyme, bile salts and pancreatin resistance

To simulate the saliva *in vitro*, 200 µL of the bacterial suspensions were inoculated in a sterile electrolyte solution-SES (0.22 g/L

Table 1. Primers used for PCR amplification of virulence genes in *Enterococcus* sp.

Primer	Sequence (5'- 3')	Ta (ºC)	bp	Reference
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	54	688	Creti et al. (2004)
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	56	375	Galli et al. (1990)
gelE	GTTCATGTCTATTTTCTTCAC CTTCATTATTTACACGTTTG	56	402	Mannu et al. (2003)
ace	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	56	320	
cpd	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	50	782	Eaton and Gasson (2001)

Ta(ºC): Annealing temperature; bp: base pairs; *cyl*A: cytolisin; *asa*1: aggregation substance; *gel*E: gelatinase; *ace*: collagen-binding protein; *cpd*: sex pheromone

$CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$) in the presence of 100 mg/L of lysozyme (Sigma-Aldrich) in accordance with Vizoso-Pinto et al. (2006). Bacterial suspensions in SES without lysozyme were included as control. Samples were incubated at 37ºC and microbial counts after 0, 30 and 120 min were carried out on MRS agar (24-48 h; 37ºC). Survival rate was calculated as percentage of the CFU/mL after 30 and 120 min in comparison to the CFU/mL at time 0.

Resistance to bile salts and pancreatin was measured as described by Charteris et al. (1998), with modifications. The overnight culture was adjusted to pH 8 and a solution of bile salts (Oxoid) was added to a final concentration of 0.3% or 0.1 g/ml of Pancreatin (Sigma). The mixture (bile salt/ bacterial cells and pancreatin/bacterial cells) was incubated for 0 and 240 min at 37°C. Aliquots were taken for determination of CFU onto the MRS agar. The plates were incubated for 48 h. The addition of bile salt was omitted in the control tube. Results were expressed as percentage of growth as compared to the control (CFU/mL at time zero).

Autoaggregation and co-aggregation assay

The extent of autoaggregation and co-aggregation in the selected probiotic isolates was assessed with the method described by Kos et al. (2003), and the percentage of autoaggregation and co-aggregation was calculated by following Mojgani et al. (2015) descriptions. As to the autoaggregation, overnight-grown cultures of the tested isolates were harvested by centrifugation and the pellet was suspended in PBS (pH 7.0) to obtain an OD (600 nm) of 0.6. The tubes were incubated at 37°C, and the absorbance at 600 nm of the celular suspensions was monitored every 1 h for a period of 5 h. Co-aggregation assay was performed by mixing equal volumes of a washed-cell suspension of selected probiotic isolates with equal volume of overnight grown cultures of *L. monocytogenes* (CDC 4555). The tubes were incubated at room temperature and absorbance at 600 nm was measured at 5 h. Controls included pure cultures of bacterial cells suspension in PBS.

Screening for enterocin production

The antimicrobial screening assay was evaluated in accordance with Ogaki et al. (2016). Enterococci strains were streaked in plates containing MRS agar, which were then incubated for 24 h at 37°C. The plates were inverted to receive 1 mL of chloroform in the covers, and remained closed for 20 min. Residual chloroform was evaporated by opening the plates. Using the pour plate method, each indicator strain (10^8 cells.mL^{-1}) was inoculated into soft MRS agar (0.8%), poured into plates forming an overlay, and these plates were incubated for 24 h at 37°C. If inhibitions zones were found around the colonies, the isolates were considered able to produce enterocin. One used indicator strains such as *Listeria innocua* CLIP 12612, *L. monocytogenes* CDC 4555, *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 25925, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538, *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium UK1 and *Escherichia coli* BAC 49LT ETEC.

Statistical analysis

Statistical analysis was carried out using the software STATISTICA 7 (StatSoft Italia, Padova, Italy). Analysis of variance test (ANOVA) was done in order to determine a significant difference of viability among *Enterococcus* strains and *L. acidophilus*. The collected data were analysed at the significance level of $p < 0.05$.

RESULTS

Of all strains, 53 were chosen based on their absence of virulence, hemolysis and antimicrobial susceptibility. Almost 2% of the strains showed resistance to vancomicyn and eritromicyn, and 54% to tetracyclin, while other strains were sensitive to all antimicrobial used.

Twenty-four strains (that is, 45%) were *in-vitro* resistant to bile salt and pancreatic enzyme, ranging from a minimum value of 81.5% to a maximum of 105 and 79.2% to 108.2, respectively (Table 2). The low pH-tolerance property of 24 *Efm* strains was investigated by culturing at pH 3.0 for 120 and 240 min. Of these, seven strains showed higher tolerance, with a survival rate greater than the control strain (*L. acidophilus*) (Table 2), in particular, the Efm 55, Efm 58, Efm 67, Efm 9A, Efm 16A, Efm 19A, Efm 44A strains.

Table 2. Tolerance of isolated strains to low pH, bile salts and pancreatic enzymes.

Isolated strain	pH tolerance		Bile salt tolerance	Pancreatic enzyme tolerance
	Survival (%) pH 3.0		Survival (%) 0.3% bile	Survival (%)
	120 min	240 min	240 min	240 min
Efm19	-	-	82.3	99.2
Efm 23	-	-	97.0	83.7
Efm 25	-	-	98.2	87.5
Efm 26	-	-	91.2	95.6
Efm 38	-	-	91.0	85.0
Efm 51	-	-	91.0	79.2
Efm 55	89.3	94.8	82.5	81.1
Efm 58	107.1	99.6	81.5	87.4
Efm 62	-	-	82.0	86.0
Efm 65	-	-	92.0	95.1
Efm 67	91.5	88.2	95.1	96.9
Efm 72	-	-	96.2	83.4
Efm 8A	-	-	83.6	80.6
Efm 9A	93.6	105.2	90.1	87.0
Efm 10A	-	-	89.9	96.7
Efm 11A	-	-	85.5	108.2
Efm 12A	-	-	102.5	83.0
Efm 13A	-	-	98.0	85.7
Efm 15A	-	-	85.2	95.4
Efm 16A	86.0	91.1	105.0	83.3
Efm 19A	98.8	97.0	101.5	90.0
Efm 20A	-	-	87.4	88.9
Efm 26A	-	-	89.5	89.9
Efm 44A	115.8	107.4	92.0	87.8
La	77.8	86.2	81.0	78.6

La: *L. acidophilus*; (-) low survival rate when compared with control bacteria.

In addition, it was studied the survival of these isolates in SES solution containing 100 mg/ml of lysozyme. The isolated strains survived in the presence of lysozyme for 30 and 120 min.

When taken together, results showed that strains Efm 55, Efm 58, Efm 67, Efm 9A, Efm 16A, Efm 19A, Efm 44A were significantly different ($p < 0.05$) in all conditions as compared with the control strain.

According to the autoaggregation results, the Efm9A, Efm19A and Efm67 strains demonstrated strong auto-aggregation phenotype, 100, 92 and 50%, respectively, within 5 h of incubation. Moreover, the Efm55, Efm58 strains showed moderate autoaggregation values (45-37%), and the Efm16A did not show any aggregation during the incubation hour. All strains exhibited co-aggregation with strain-pathogen (*L. monocytogenes*), showing values among 65 to 78%.

The antimicrobial spectra of *Enterococcus* strains were investigated by using 9 pathogens as targets. The isolated strains demonstrated broad activity against all tested Gram-positive (*L. innocua* CLIP 12612, *L. monocytogenes* CDC 4555, *E. faecalis* ATCC 29212, *S. aureus* ATCC 25925, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538) and Gram-negative (*S.* Enteritidis ATCC 13076, *S.* Typhimurium UK1) strains, with halos ranging from 0.4 to 1.52 mm. Although that *E. coli* BAC 49LT ETEC was not inhibited by *Enterococcus* strains.

DISCUSSION

Among the Lactic Acid Bacteria (LAB), members of the *Enterococcus* genus have been object of increasing scientific work, because of its wide range of health-promoting effects. The commonly accepted criteria is that probiotic organisms should be resistant to acid and bile, which are elements present in the stomach and small intestine conditions. In our previous work, the *E. faecium* demonstrated high ability to survive in the presence of lysozyme and pancreatic enzymes, bile salt and low pH, during several hours. More importantly, none of the *E. faecium* strains carried the virulence factors cylA and

cylB, required in hemolytic activity, which is the most important virulence trait that lyses the eukaryotic cells (Kayser, 2003). *E. faecium* also showed low antimicrobial resistance, though antimicrobial-resistant probiotics can be used in combination with antimicrobial agents (Sanders et al., 2010).

Based on cell growth /survival, we selected seven *E. faecium* strains for investigation. These strains, initially named as Efm 55, Efm 58, Efm 67, Efm 9A, Efm 16A, Efm 19A, Efm 44A, presented significant activity when compared with the control bacteria.

E. faecium is found in many food products, especially those from animal origin, such as dairy products (Foulquié Moreno et al., 2006; Kivanç et al., 2016). They are most frequently present in many traditional cheeses – prepared mostly from raw ewes' or goats' milk –, and play an important role in the ripening of such products (Manolopoulou et al., 2003). A high prevalence of enterococci in processed foods may be attributed to their resistance to heat, extreme salinity and harsh conditions during the ripening of fermented foods (Gomes et al., 2008; Jurkovic et al., 2006). Altogether, enterococci strains have been a promising probiotic in the promotion of human and animal health by improving the intestinal microbial balance (Foulquié-Moreno et al., 2006; Franz et al., 2011; Buntin et al., 2008).

In this study, Efm strains were exposed to pH 3.0 for 240 min, and several strains were highly resistant to pH 3.0 with levels that were higher than the control bacteria. The average time food stays in the stomach is 3 h, and, in general, our results meet those of other researchers (Mansour et al., 2014).

Once bacteria have survived the gastric barrier (low pH), the environment in the small intestine is a second major barrier for probiotic strains. Therefore, authors have recommended testing bacterial resistance to bile salt concentrations in the 0.3% and pancreatin 0.1 mg/mL to the selection of probiotic bacteria for human use (Bezkorovainy, 2001; Tuomola et al., 2001; Mansour et al., 2014). The major factors determining the survival of LAB include particular characteristics of the strains, tolerance to acid and bile, and resistance to gastric and intestinal juices (Succi et al., 2005). Amaral et al. (2017) and Sun et al. (2010) showed that *E. faecium* was more stable during the simulation of the gastrointestinal tract, showing greater cell viability.

High acidity and high concentration of bile components in the gastrointestinal tract influence the selection of potential probiotic strains (Hyronimus et al., 2000). However, small intestine tolerance is potentially more important than gastric survival. With the development of new delivery systems and the use of specific foods, some studies indicate that acid-sensitive strains can be buffered through the stomach. However, in order to promote a positive effect in the host, probiotics need to survive and colonize his/her small intestine, and the condition of such environment may be an essential criterion for future probiotics (Huang and Adams, 2004).

This study investigated the antibacterial activity of *E. faecium* strains isolated from soft cheese. These *E. faecium* strains were able to inhibit *L. innocua*, *L. monocytogenes*, *E. faecalis*, *S. aureus, and Salmonella.* In particular, *E. coli* was not sensitive to all *E. faecium* strains.

Besides determining that enterococci strains showed high auto-aggregation, one has also demonstrated that they exhibit high co-aggregation against *L. monocytogenes* strain. Aggregation and co-aggregation among bacteria play an important role in the prevention of surface-colonization by pathogens (García-Cayuela et al., 2014), as it is well known that the co-aggregation abilities of LAB strains might interfere with the ability of pathogenic species to infect the host, and can also prevent the colonization of food-borne pathogens (García-Cayuela et al., 2014).

In summary, the results obtained in this study suggest that *E. faecium* strains are resistant to pass through the gastrointestinal tract. One also verified the viability of this strain through the exposure rate and the combination of simulated gastric juice and bile salts, intestinal juice, bile and acid tolerance. Further investigations may be warranted to elucidate its potential health benefit and its application as a promising probiotic strain in the food industry.

Conclusion

This study have demonstrated that *E. faecium* strains of soft-cheese origin may be a probiotic candidate with functional characteristics in terms of resistance to low pH and bile salts, survival under digestion conditions and adhesion, antimicrobial properties, antibiotic resistance, and presence of the virulence factors as well as hemolytic reaction. Further work is in progress to characterize both the bacteriocin(s) and its probiotic functionality.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Phenotypic and genotypic detection of macrolide-lincosamide-streptogramin B resistance among clinical isolates of *Staphylococcus aureus* from Mansoura University Children Hospital, Egypt

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Widespread use of Macrolide, lincosamide and Streptogramin B (MLSB) family of antibiotics in the treatment of *Staphylococcus aureus* (*S. aureus*) has led to an increased resistance to MLSB antibiotics. The purpose of this study was to determine the frequency of MLSB resistance among clinical isolates of methicillin sensitive *S. aureus* (*MSSA*) *and Methicillin resistant S. aureus* (*MRSA*) from Mansoura University Children Hospital (MUCH), Egypt, phenotypically by using D-test and genotypically by detection of *erm* genes by PCR. Different microbiological samples were collected under complete aseptic condition from patients in MUCH according to the site of infection over a period of 9 months from March 2016 to November 2016. *S. aureus* isolates were identified using standard microbiological methods. MRSA was detected by growth on oxacillin screen agar plate and cefoxitin disk screen test. Antimicrobial susceptibility of the isolates was determined by Kirby-Bauer disk diffusion method. *S. aureus* isolates that were found to be erythromycin resistant were further studied for inducible clindamycin resistance using D-zone test according to CLSI recommendations. *erm* genes in *S. aureus* isolates were detected by PCR. Among 230 *S. aureus* isolates, 164 were MSSA (71.3%) and 66 were MRSA (28.7%). Twenty-five MSSA (15.2%), and 37 MRSA (56.1%) isolates were erythromycin resistant. Constitutive MLSB phenotype (cMLSB) (30.3 and 4.2%) and inducible MLSB phenotype (iMLSB) (22.7 and 7.9%) were observed in MRSA and MSSA, respectively by D-zone test. The rate of iMLSB phenotype and cMLSB phenotype in MRSA was significantly higher than in MSSA isolates. The frequency of *erm*A, *erm*B and *erm*C genes were 72.9, 5.4 and 13.5% in MRSA isolates and 60, 4 and 12% in MSSA isolates, respectively. Screening test for of iMLSB-resistant strains is very important by double disk diffusion test (D-test). This phenotypic test is simple, accessible and reliable method that can be done in every laboratory and research facility, without the need of costly genetic tests. Since the treatment of patients infected with *S. aureus* with iMLSB phenotype with clindamycin can lead to the expansion of constitutive resistance (cMLSB) and therapy failure.

Key words: Clindamycin, *cMLSB*, erm genes, iMLSB phenotype, MRSA, MSSA, *S. aureus*.

INTRODUCTION

Methicillin resistance in *Staphylococcus aureus* (*S. aureus*) is an increasing problem in children and adult populations. MRSA is resistant to almost all beta-lactam antibiotics. Resistance to other antibiotics is also common, especially in hospital-acquired MRSA (Valle et al., 2016). Initially, MRSA was linked to infections associated to health care (hospital-acquired MRSA). Currently, MRSA represent a major problem in the community (Community-associated MRSA)(Nascimento et al., 2015). While the community-associated MRSA diseases are related to skin infections, the more severe clinical infections are more frequently related to hospitalized patients (Baddour et al., 2006).

Emergence of MRSA, has led to the enquiry of possible other antibiotics other than beta-lactam for staphylococcal infections treatment as erythromycin, clindamycin, gentamicin and ciprofloxacin (Valle et al., 2016). Macrolide (erythromycin), lincosamide (clindamycin) and Streptogramin B (MLSB) family of antibiotics is generally used in the treatment of staphylococcal infections; clindamycin is a good alternative in penicillin allergic patients in treatment of *S. aureus* infections. In addition, clindamycin has excellent oral bioavailability making it a good option for outpatient therapy and substitution after intravenous antibiotics. However, this widespread use has resulted in an increase in the number of *Staphylococci* strains resistant to MLSB antibiotics (Gherardi et al., 2009).

S. aureus and *MRSA* resistance to Macrolide antibiotic may be due to an active efflux mechanism encoded by *msrA* (encoding resistance to macrolides and Type B streptogramins only) or ribosomal target modification affecting macrolides, lincosamides, and Type B streptogramins (MLSB resistance) encoded by erm genes (Navaneeth, 2006). Three main *erm* (erythromycin ribosome methylation) genes, that is, *erm*(A), *erm*(B) and *erm*(C), have been defined in *Staphylococci*. They encode enzymes for inducible or constitutive resistance to MLSB agents through methylation of the 23S ribosomal RNA, thus reducing binding by MLSB agents to the ribosome (Martineau et al., 2000). *In vitro*, *S. aureus* isolates with constitutive resistance (cMLSB) are resistant to erythromycin and clindamycin, and isolates with inducible resistance (iMLSB) are resistant to erythromycin but appear to be susceptible to clindamycin. The risk for therapeutic failure is increased as cMLSB may rise from iMLSB during the course of clindamycin therapy in patients with severe *Staphylococci* infections Goudarzi et al., 2016).

Constitutive resistance can be readily detected, but inducible resistance is not detectable by routine antimicrobial susceptibility tests (Martineau et al., 2000).The double-disk diffusion test (D test) was recommended by Clinical and Laboratory Standards Institute (CLSI) as phenotypic method to screen for inducible resistance (CLSI, 2013). *erm*A, *erm*B and *erm*C among clinical isolates of *S. aureus* is detected by polymerase chain reaction (PCR) with specific primers as a genotypic method to confirm the presence of the MLSB genes. The purpose of our study was to determine the frequency of macrolide-lincosamide-streptogramin B (MLSB) resistance among clinical isolates of MSSA *and MRSA* from Mansoura University Children Hospital, Egypt, phenotypically by using D‑test and genotypically by detection of erm genes by PCR.

MATERIALS AND METHODS

Isolation and identification of *S. aureus*

Different microbiological samples (wound swabs, pus, blood, urine, respiratory tract samples and fluid) were collected under complete aseptic condition from patients in MUCH according to the site of infection over a period of 9 months from March 2016 to November 2016. The samples were transported and processed in Microbiology Diagnostic and Infection Control unit (MDICU) in Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University. Samples were inoculated on 5% sheep blood agar and Mac Conkey's agar (Oxoid, UK), incubated at 37°C for 24-48 h, and examined for bacterial growth.

S.aureus isolates were identified by conventional biochemical tests (catalase, coagulase, DNase) and commercial identification system (API-STAPH; bioMérieux, Fance) (Gupta et al., 2009). Identical isolates from the same patient were not included in the study.

Detection of MRSA

MRSA was detected by growth on oxacillin screen agar plate containing 6 µg/ml of oxacillin in Mueller-Hinton agar supplemented with 4% NaCl and by cefoxitin disk screen test, using a 30 µg cefoxitin disc (Oxoid, UK). An inhibition zone diameter of ≤ 21 mm was reported as oxacillin or methicillin resistant and a zone diameter of ≥ 22 mm was considered sensitive according to the CLSI guidelines (CLSI, 2013).

Antimicrobial susceptibility testing

Kirby-Bauer disk diffusion method was used to determine Antimicrobial susceptibility of the isolates according to CLSI

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Figure 1. D-shape zone of growth inhibition around clindamycin disk (iMLSB phenotype).

guidelines. Briefly a 0.5 McFarland suspension of bacteria was prepared and inoculated on Mueller-Hinton's agar plates (Oxoid, UK). The following antibiotic disks were used; penicillin (10U), amoxicillin-clavulanic acid (20/10 µg) cefoxitin (30 µg), gentamicin (10 µg), clindamycin (2 µg), erythromycin (15 µg), trimetoprim-sulfametoxazol (1.25/23.75 µg), ciprofloxacin (5 µg), tetracycline (30 ug) and rifampin (5 µg) (CLSI, 2013).

Vancomycin and oxacillin minimal inhibitory concentrations (MICs) were determined by E-Test (Bio Mérieux) according to CLSI guidelines.

S. aureus ATCC 25923 and *S. aureus* ATCC 29213 were used as standard strains and quality control for disk diffusion and MIC tests; respectively.

Disk approximation test with erythromycin and clindamycin (D-Zone test)

Erythromycin resistant *S. aureus* isolates were further studied for inducible clindamycin resistance by disk approximation test with erythromycin and clindamycin (D-zone test) according to CLSI guidelines. 0.5 McFarland suspensions was prepared from overnight growth of erythromycin resistant *S. aureus*. Then inoculated and spread over the surface on Mueller-Hinton agar plates (Merck, Germany). One erythromycin disk (15 µg) and one clindamycin disk (2 µg) were placed 15 mm distance from each other on the inoculated plates. Plates were incubated at 35°C and read after 18 h (Cetin et al., 2010).

According to the inhibition zone diameters, the isolate was considered to be:

1) Macrolide-lincosamides streptogramin B inducible phenotype (iMLSB) (D test positive); if the isolate was erythromycin resistant and exhibited D-shaped inhibition zone around the clindamycin disc, (Figure 1).
2) Macrolide-lincosamides streptogramin B constitutive phenotype (cMLSB); if the isolate was resistant to both erythromycin and clindamycin.
3) Negative for inducible resistance (D test negative), but to have an active efflux pump (MSB); if the isolate was erythromycin resistant and clindamycin susceptible, with both zones of inhibition showing a circular shape (Bannerman et al., 2007).

DNA extraction

DNA was extracted from MRSA and MSSA isolates with macrolide-lincosamide-streptogramin (MLS) resistance using QIAamp® DNA Mini kits, QIAGEN (Germany) according to the producer's guidelines.

Table 1. Primers used in this study.

Gene	Primers sequence (5' →3')	Product size (Pb)
ermA	TATCTTATCGTTGAGAAGGGATT CTACACTTGGCTTAGGATGAAA	139 bp
ermB	CTATCTGATTGTTGAAGAAGGATT GTTTACTCTTGGTTTAGGATGAAA	142 bp
ermC	CTTGTTGATCACGATAATTTCC ATCTTTTAGCAAACCCGTATTC	190 bp

PCR for detection of *erm* genes

erm genes were amplified by PCR using specific primers for the *erm A*, *B* and *C* genes as exhibited in Table 1. Each reaction was performed in a final volume of 25 µL consisting of 5 µL of DNA template, 2.5 µL of PCR buffer (×10), 1 µL $MgCl_2$ (50 mM), 0.5 µL of dNTPs (10 mM), 5 µM of each *erm*A, *erm*B and *erm*C forward and reverse primers, 0.25 µL of Taq DNA polymerase (5 u/µL), 11.25 µL distill water.

PCR was achieved with the following reaction conditions: Initial denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min (Coutinho et al., 2010).

Amplicons were analyzed after running on 2% agrose gel containing ethidium bromide in comparison to 50 bp molecular size standard ladder (Thermo Scientific Inc.).

Statistical analyses

Descriptive data were presented as frequencies and percentages via SPSS software version 18. Chi-square test was used to determine any significant differences between prevalence of the tested genes among *S. aureus* and MRSA strains. P value ≤ 0.05 was considered statistically significant.

Ethical Issues

This study was approved by Mansoura Faculty of Medicine, Egypt ethical committee (No: R/ 16.07.25). Written Informed consent was obtained from the guardian of each participant child. Privacy and confidentiality of personal information were saved and protected.

RESULTS

Two hundred and thirty (230) *S. aureus* isolates from different clinical samples were included in our study. 164 were MSSA (71.3%) and 66 were MRSA (28.7%).

MSSA and MRSA were most frequently isolated from Pus (26.9%), wound swab (26.1%), followed by blood culture (13.04) (Table 2). Twenty-five MSSA (15.2%), and 37 MRSA (56.1%) isolates were erythromycin resistant.

Table 2. Distribution of MSSA and MRSA isolates in different clinical samples.

Specimen	MSSA		MRSA		Total	
	No.	%	No.	%	No.	%
Pus	50	30.5	12	18.2	62	26.9
Wound swab	45	27.4	15	22.7	60	26.1
Blood culture	20	12.2	10	15.2	30	13.04
Catheter	5	3.04	8	12.1	13	5.6
Urine culture	10	6.1	8	12.1	18	7.8
Respiratory tract sample	12	7.3	6	9.09	18	7.8
Eye swab	3	1.8	2	3.03	5	2.2
Ear discharge	7	4.3	3	4.5	10	4.3
Fluid	5	3.04	0	0	5	2.2
others	7	4.3	2	3.03	9	3.9
Total	164	100	66	100	230	100

MRSA = methicillin-resistant *Staphylococcus aureus*; MSSA= methicillin- sensitive *Staphylococcus aureus*.

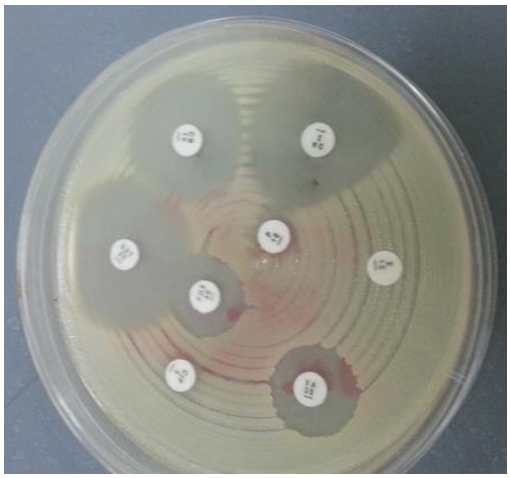

Figure 2. Antibiotic sensitivity of MRSA with positive D-Zone test.

Clinical isolates that displayed erythromycin resistance were tested for inducible resistance by D test (Figure 2).

Antimicrobial resistance rate to gentamicin, trimetoprim-sulfametoxazole, ciprofloxacin and clindamycin showed statistically high significant differences between MRSA and MSSA isolates. Also, no antibiotic resistance was observed against vancomycin in both MRSA and MSSA (Table 3).

Regarding D-zone test, cMLSB phenotype (30.3 and 4.2%), iMLSB phenotype (22.7 and 7.9%) and MS phenotype (3.3 and 3.04%) were observed in MRSA and MSSA, respectively (Table 4).

The rate of iMLSB phenotype and cMLSB phenotype was significantly higher in MRSA isolates; *P* value = 0.007 and *P* <0.001, respectively.

The frequency of *erm*A, *erm*B and *erm*C genes detected in MRSA and MSSA isolate were 72.9, 5.4, 13.5% and 60, 4, 12%, respectively (Table 5).

Distribution of *erm* genes among different MLSB phenotypes is as shown in Table 6.

DISCUSSION

Antimicrobial resistance is a worldwide problem, particularly among hospital acquired pathogens. Staphylococci have become one of the most common causes of both hospital acquired and community acquired infection (Navaneeth et al., 2006).

The increasing prevalence of methicillin resistance among Staphylococci resulted in renewed interest in the usage of Macrolide-Lincosamide-Streptogramin B (MLSB) antibiotics to treat *S. aureus* infections (Gupta et al., 2009).

Nevertheless, extensive usage of MLS B antibiotics has led to an increase in the number of Staphylococcal strains acquiring resistance to MLSB antibiotics (Cetin et al., 2010).

In the current study, 27.8% isolates were found to be MRSA that is comparable with a study conducted in Iran by Seifi et al. (2012). On contrary, higher result (48%) was reported by Ghanbari et al. (2016).

In the present study, erythromycin resistance was detected in 56.1 and 15.2% of MRSA and MSSA isolates, respectively. These results are in accordance with previous other studies (Ciraj et al., 2009; Prabhu et al., 2011).

In our study, cMLSB phenotype predominated over iMLSB phenotype in MRSA isolate (30.3% vs. 22.7%) that is similar to the finding of Gadepalli et al. (2006) and Dardi and Khare (2013).

Constitutive and inducible resistance clindamycin resistance phenotype was significantly higher in MRSA

Table 3. Antibiotic resistance rate among MSSA and MRSA.

Antibiotic	MSSA (n=164)		MRSA (n=66)		P
	No	(%)	No	%	
Penicillin	150	91.4	66	100	0.67
Oxacillin	0	0	66	100	<0.001
Cefoxitin	0	0	66	100	<0.001
Amoxicillin-clavulanic acid	24	14.6	66	100	<0.001
Gentamicin	40	24.3	35	53	0.004
Trimetoprim-sulfametoxazole	25	15.2	28	42.4	0.001
Tetracycline	15	9.1	20	30.3	0.001
Ciprofloxacin	30	18.3	40	66.7	<0.001
Rifampin	5	3.04	1	1.5	1.00
Clindamycin	10	6.1	22	33.3	<0.001
Erythromycin	25	15.2	37	56.1	<0.001
Vancomycin	0	0	0	0	-

MRSA: Methicillin resistant *S. aureus*, MSSA: Methicillin sensitive *S. aureus;* R: resistant. S: sensitive; P value <0.05 is considered as statistically significant.

Table 4. MLSB resistance phenotypes in MSSA and MRSA.

Parameter	MSSA (n=164)	MRSA (n=66)	P
Constitutive MLSB resistance	7 (4.2%)	20 (30.3%)	<0.001
Inducible MLSB resistance	13 (7.9%)	15 (22.7%)	0.007
MS Phenotype	5 (3.04%)	2 (3.3%)	1.00

MRSA: Methicillin resistant *S. aureus*, MSSA: Methicillin sensitive *S. aureus*. P value <0.05 is considered as statistically significant.

Table 5. Distribution of *erm* genes among macrolide-resistant MSSA and MRSA isolates.

Isolate	Genotype			
	ermA	***ermB***	***ermC***	***ermA+ ermC***
MSSA (25)	15 (60%)	1 (4%)	3 (12%)	1 (4%)
MRSA (37)	27 (72.9%)	2(5.4%)	5 (13.5%)	0(0%)

Table 6. Distribution of *erm* genes among different MLSB phenotypes.

MLS phenotype	Genotype			
	ermA	***ermB***	***ermC***	***ermA+ ermC***
iMLSB				
MSSA(13)	9	-	1	1
MRSA(15**)**	10	1	3	0
cMLSB				
MSSA (7)	6	1	2	0
MRSA(20**)**	16	2	2	0
MLS				
MSSA (5)	**-**	**-**	**-**	**-**
MRSA (2)	**-**	**-**	**-**	**-**

isolate as compared to MSSA isolate. Similar results were reported in other studies (Prabhu et al., 2011; Gadepalli et al., 2006; Dardi and Khare, 2013; Mahesh et al., 2013; Memariani et al., 2009). However, Schreckenberger et al. (2004) showed higher percentage of inducible resistance in MSSA as compared to MRSA.

Regarding MS phenotype, there was no statistical significance between MRSA and MSSA isolates. Erythromycin resistance in Staphylococci is encoded by *erm* genes. The frequency of *erm* A, *erm* B, *erm* C in MRSA and MSSA were 72.9, 5.4, 5% and 60, 4, 12%, respectively.

The frequency of *erm* genes is variable in different studies. In our study, *erm A* was the most frequent gene detected in MRSA (72.9%) and MSSA isolate (60%); this in accordance with study conducted by Saderi et al. (2011). Contrary to our result, *erm* C was the most common gene detected in other studies conducted in Iran, Turkey and Brazil (Ghanbari et al., 2016; Aktas et al., 2007; da Paz Pereira et al., 2016). Also, Zmantar et al. (2011) reported *erm* B was the most common genes detected from *S. aureus* isolates.

In accordance with another study conducted by Otsuka et al. (2007), our study showed that phenotypic method by D-test and genotypic detection of *erm genes* was in parallel for detection of macrolide resistance in *S. aureus*.

Conclusion

Since treatment of *S. aureus* infections with iMLSB phenotype by clindamycin can lead to the expansion of constitutive resistance (cMLSB) and therapy failure, screening test for iMLSB‑resistant strains is very essential by double disk diffusion test. This phenotypic test is a simple, accessible and reliable method that can be done in every laboratory and research facility, without the need of costly genetic tests.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibacterial effects of *Anogeissus leiocarpus* (DC.) Guill. & Perr. and *Terminalia glaucescens* Planch. Ex Benth. on rapidly growing mycobacteria species

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The development of effective and less toxic antimicrobial agents is required for the treatment of respiratory tract infections. This study was carried out to evaluate the phytochemical and antibacterial activities of *Anogeissus leiocarpus* (DC.) (Guill. & Perr.) and *Terminalia glaucescens* (Planch. ex Benth.) against non-tuberculous mycobacteria species. The methanol, dichloromethane and aqueous extracts were screened against five (5) non-tuberculosis mycobacteria (NTM) species by agar diffusion method. Minimum inhibitory concentration (MIC) was determined by agar dilution method while bactericidal studies were done by viable count technique. The methanol and aqueous extracts were active against all the test organisms with zones of inhibition ranging from 10±0.0 to 25±0.5 mm. The MIC and MBC range from 0.3125 to 2.5 and 1.25 to 10 mg/mL, respectively. Bactericidal activities of aqueous extracts against *Mycobacterium smegmatis* ATCC 19420 revealed a drastic dose-dependent decline in the surviving population after 6 h of exposure accompanied by a total (100%) kill after 24 h of exposure. The antimicrobial activities demonstrated by these plants suggest the presence of therapeutically important antimycobacterial compounds and thus justify as well as support the use of these medicinal plants for the treatment of respiratory tract infections.

Key words: *Anogeissus leiocarpus* (DC.) (Guill. & Perr.), *Terminalia glaucescens* (Planch. ex Benth.), antibacterial, nontuberculous mycobacteria species, bactericidal, *in vitro.*

INTRODUCTION

Rapidly growing mycobacteria (RGM) belong to non-tuberculous mycobacteria group, which is a heterogeneous group of organisms that occasionally are a primary cause of lung diseases and affect patients with underlying chronic lung disease such as bronchiectasis, pneumoconiosis, or healed tuberculosis (Griffith et al., 2007). RGM pulmonary infection is serious and difficult to cure, with improvement or resolution in less than one-half of patients with cancer who have definitive/probable infection. A common feature of all RGM is their resistance

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to first-line anti-tuberculous agents hence the need for new therapeutic agents that will be effective for the cure of RGM infections. The use of medicinal plants for the treatment of infectious diseases is an age long practice that is on the rise globally. Thus, two medicinal plants used in folklore medicine for the treatment of cough and tuberculosis identified from an ethnobotanical survey were chosen for investigation of their anti-mycobacterial activity. The plants are *Anogeissus leiocarpus* (DC.) Guill. & Perr. (African birch) and *Terminalia glaucescens* Planch. ex Benth. (Combretaceae).

Anogeissus is a genus of trees native to South Asia, the Arabian Peninsula, and Africa, belonging to family Combretaceae. The genus has eight species, five native to South Asia, two endemic to the southern Arabian Peninsula, and one native to Africa (Mann et al., 2008). *A. leiocarpus* is found in Africa from northeastern Ethiopia to Senegal, and its bark is used to produce Anogelline, a substance used in cosmetics (Shuaibu et al., 2008). Traditionally, in most parts of Hausa land (Northern Nigeria), infusion of *A. leiocarpus* leaves is used in the treatment of cough, wound infections and rashes in small children (Bizimana, 1994). *A. leiocarpus* is traditionally acclaimed to be effective in treating infectious wounds in man and animals (Dweek, 1997). The pulped roots are applied to wounds and ulcers, and the powdered bark is also rubbed on gums to reduced tooth ache. It is also used as vermifuges and the leaves decoction is used for washing and fumigation (Ibrahim et al., 1997, 2005). The root of the plant when used as chewing stick is known to have antibacterial effects on *Lactobacillus* sp. (Owoseni and Ogunnusi, 2006). Antimicrobial activity against a variety of viruses, the malaria parasite and some bacteria has also been demonstrated (Taiwo et al., 1999). *A. leiocarpus* is used medically for the treatment of diabetic ulcers, ascariasis, gonorrhoea, general body pain, blood clots, asthma, coughing, bronchitis, pulmonary disorder, hemoptysis, pneumonia, catarrh, hay-fever and tuberculosis (Mann et al*.,* 2003, 2007, 2008; Barku et al., 2013). Personal interactions with some herb sellers revealed that leaf extracts of this tree are commonly used in the treatment of typhoid fever, diarrhoea, malaria fever, rheumatism, cough and skin infections whether administered singly or in combination with other herbs.

Terminalia is a genus of large trees of the flowering plant family Combretaceae, comprising around 100 species distributed in the tropical regions of the world (Mann et al., 2008). It is traditionally used in the treatment of diabetes (Njomen et al., 2008; Ndukwe, 2005). It is also widely used as a chewing stick in Nigeria, thus various studies have been carried out on its antimicrobial activity against oral pathogens (Ogundiya et al., 2008). *Terminalia glaucescens* is used in the treatment of dysentery. It has found use as an antimicrobial agent in the last stages of AIDS (Koudou et al., 1995). Antiplasmodial activity of ethanolic extract of the plant was described by Mustofa et al. (2000). Trees of this genus are known to be a source of secondary metabolites, e.g. cyclic triterpenes and their derivatives, flavonoids, tannins and other aromatics. Some of these substances have antifungal, antibacterial, anti-cancer and hepatoprotective effects.

In this study, the inhibitory activities of *A. leiocarpus* (DC.) (Guill. & Perr.) (leaf, stem bark and root) and *T. glaucescens* (Planch. ex Benth.) (root) on rapidly growing mycobacteria were investigated, to justify the use of these plants in the treatment of respiratory tract infections, especially tuberculosis.

MATERIALS AND METHODS

Plant collection, extraction and preparation of extracts

A. leiocarpus (leaf, stem bark and root) and *Terminalia glaucescens* (root) plant materials were obtained and identified according to the international WHO guidelines "WHO Guidelines on Good Agricultural and Collection Practices" (WHO, 2003). Voucher specimens were deposited at Forest Research Institute of Nigeria Ibadan, Oyo State (FRIN), where the plants species were identified, authenticated and assigned voucher specimen number FHI 109925 and FHI 108282 for *Anogeissus leiocarpus* and *Terminalia glaucescens,* respectively**.** Plant materials were soaked in methanol and extracted for 72 h with constant agitation. The extraction process was repeated thrice. Extracts were filtered using Whatman No. 1 filter paper and combined, prior to being concentrated to dryness using a rotary evaporator. Fifty grams (50 g) of methanol extracts from the samples was defatted with *n*-hexane and partitioned into dichloromethane and water. The methanol extract (ME), dichloromethane (DCM) and aqueous (AQ) partitions were dried using a rotary evaporator, weighed and stored at 4°C. Solutions of extracts/partitions were reconstituted in 40% methanol with final concentrations of 1, 2, 10 and 20 mg/mL for the initial screening. Concentrations in the range of 0.156 and 20 mg/mL were also prepared to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the bioactive extracts.

Antimicrobial agents

The chemotherapeutic agent used in the test as a positive/drug control was Rifampicin at 20 and 40 µg/mL (Nicholas Laboratories Limited, England), while the negative/solvent control was 40% methanol.

Phytochemical screening

Phytochemical screening was carried out to detect the presence of secondary metabolites such as anthraquinones, flavonoids, tannins, saponins, alkaloids, phenol among others using methods described by Harborne (1998).

Strains of nontuberculous mycobacteria (NTM)

Five non-tuberculous mycobacteria isolates used for this investigation were *Mycobacterium fortuitum* ATCC 684*, Mycobacterium smegmatis* ATCC 19420, *Mycobacterium phlei* ATCC 19240, *Mycobacterium smegmatis* and *Mycobacterium abscessus*.

Table 1. Extraction yields of *A. leiocarpus* and *T. glaucescens*.

Plant	Plant Part	Weight of powdered sample (g)	Solvent	Yield (g)	Percentage yield
A. leiocarpus	Leaf	213	Methanol	5.03	2.36
			Dichloromethane	4.37	2.05
			Aqueous	0.63	0.29
	Stem bark	1082	Methanol	40	3.69
			Dichloromethane	0.43	0.04
			Aqueous	67.29	6.22
	Root	908	Methanol	30	3.30
			Dichloromethane	0.53	0.06
			Aqueous	52.1	5.74
T. glaucescens	Root	1057	Methanol	12.41	1.17
			Dichloromethane	1.32	0.12
			Aqueous	18.68	1.77

Susceptibility testing

Susceptibility was determined using the agar cup diffusion technique as previously described (Lawal et al., 2014). Plates were incubated at 37°C for two to three days after which diameters of zones of inhibition (mm) were measured. Methanol (40%) was included in each plate as a solvent control while Rifampicin (20 and 40 µg/mL) was used as positive control.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) of bioactive extracts were determined by a modification of standard agar dilution method procedures as previously described (CLSI, 2008). Extracts were tested at various concentrations ranging from 20 to 0.156 mg/mL. The positive/drug control was rifampicin. The MICs were determined after two to three days of incubation at 37°C. The MIC was regarded as the lowest concentration that prevented visible growth of test organisms. The experiments were performed in duplicate.

Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) of active plant extracts was determined by a modification of the method of Aibinu et al. (2007). The lowest concentration that prevented bacterial growth after 48 h of incubation was recorded as the minimum bactericidal concentration (MBC). The entire tests were carried out in duplicates to ensure accuracy. Agar plates without extracts and another agar plate without any inoculated organism were also incubated to serve as organism and extract control plates, respectively. Minimum bactericidal concentration (MBC) was also determined for the drug control (Rifampicin).

Determination of bactericidal activity

The viable counting technique previously described (Ogudo et al., 2014) was employed for this purpose. *Mycobacterium smegmatis* ATCC 19420 was used for this experiment. The procedure was carried out in duplicate. Control plates for negative and positive controls were also incubated. Plates were incubated at 37°C for 24 h before counting the colonies. The numbers of surviving bacterial cells per mL were calculated by taking into consideration the dilution factor and the volume of the inoculum. All the procedures were performed with concentrations equivalent to MIC, 2 × MIC and 4 × MIC. A graph of viable count (Log10) against time (hour) was plotted to show the rate of kill of the test organisms.

RESULTS AND DISCUSSION

The extraction yields for *A. leiocarpus* leaf, stem bark and root as well as *T. glaucescens* root are presented in Table 1. Phytochemical screening of the powdered plant samples, aqueous and dichloromethane extracts of *A. leiocarpus* and *T. glaucescens* revealed the presence of saponins, flavonoids, tannins, alkaloids, reducing sugar, glycosides and resins in the powdered samples and aqueous partitions (Table 2). The methanol and aqueous extracts were active against all the test organisms with diameter of zone of inhibition ranging between 10±0.0 and 25±0.5 mm (Tables 3 and 4), while the dichloromethane extracts had little/no activity (results not shown). The diameter of the zone of inhibition was concentration-dependent (increased with increase in the concentration of extracts) as shown in Tables 3 and 4. Most of the phytochemical components were absent in the dichloromethane extracts. This explained the reason for the aqueous extracts been more effective than the dichloromethane fractions. Since the dichloromethane extracts had little or no activity on the test organisms they were not studied further. The mechanism of inhibition of the phytochemical components on the mycobacteria species may be due to the impairment of various enzyme systems such as those involved in energy production as well as the interference with the integrity of the cell membrane and structural component synthesis (Huang and Chung, 2003; Okwu and Morah, 2007).

It was also observed that the drug control (Rifampicin) was active on all the isolates tested with the diameter of the zones of inhibition ranging from 15±0.0 to 23 ± 0.0

Table 2. Phytochemical analysis of *A. leiocarpus* and *T. glaucescens*.

Phytochemical components	*Terminalia glaucescens* root			*Anogeissus leiocarpus* root			*Anogeissus leiocarpus* leaf			*Anogeissus leiocarpus* stem bark		
	Powdered sample	Aqueous extract	DCM extract	Powdered sample	Aqueous extract	DCM extract	Powdered sample	Aqueous extract	DCM extract	Powdered sample	Aqueous extract	DCM extract
Saponins	+	+	-	+	+	-	+	+	-	+	+	-
Flavonoids	+	+	-	+	+	-	+	-	-	+	+	-
Tannins	+	+	+	+	+	-	+	+	+	+	+	+
Phlobatannins	+	-	-	+	-	-	+	-	+	+	-	+
Steroids	+	+	-	+	+	-	-	-	-	-	-	-
Cardiac glycosides	-	-	-	+	+	-	-	-	-	-	-	-
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+
Reducing sugar	+	-	+	+	+	+	+	+	+	+	+	+
Phenols	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	+	+	-	+	-	+	-	-	-	-	-	+
Glycosides	+	+	+	+	+	+	+	+	+	+	+	+
Resins	+	+	+	+	+	-	+	-	+	+	-	+

+ = Present; - = absent.

mm (Tables 3 and 4). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ranged from 0.3125 to 2.5 and 1.25 to 10 mg/mL, respectively (Table 5). The phytochemical components detected in the plant samples have been associated with antimicrobial activity (Marjorie, 1999; Mahajan and Badgujar, 2008) and could be responsible for the observed effects. The inhibitory activities of *A. leiocarpus* and *T. glaucescens* observed in this study are in agreement with earlier studies reporting the antimicrobial activities of *A. leiocarpus* and *T. glaucescens* (Batawila, 2005; Barku et al., 2013). The plants are used in folklore medicine for the treatment of diabetic ulcers, general body pain, blood clots, asthma, coughs and tuberculosis. Hollist (2004) and Taiwo et al. (1999) reported that both plants are sold as chewing sticks for the prevention or treatment of oral infections in southwest Nigeria. *T. glaucescens* is one of the plants used in the preparation of the “wonder cure” concoction used in the treatment of tuberculosis in Nigeria. The activity of the plant extracts on *Mycobacterium tuberculosis* was reported by Adeleye et al. (2008).

Bactericidal activities of aqueous extracts of *A. leiocarpus* and *T. glaucescens* on *Mycobacterium smegmatis* ATCC 19420 showed a bactericidal activity dependent on the time of exposure and the concentration of each of the extracts as shown in Figures 1 to 4. It was shown that there is a drastic dose-dependent decline in the surviving population after 6 h of exposure accompanied by a total (100%) kill after 24 h of exposure to the aqueous extracts of *A. leiocarpus* and *T. glaucescens* at doses equivalent to MIC, 2 × MIC and 4 x MIC (Figures 1 to 4). This result is similar to the kinetics study of the *in vitro* activities of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric) rhizomes against non-tuberculous mycobacteria species (Ogudo et al., 2014). The slightly high MIC values (0.31 to 2.5 mg/mL) recorded and the lower kill rate observed for these organisms as compared to the drug control-Rifampicin (5 to 20 µg/mL) in this study could be explained by the report of Nessar et al. (2012). They observed that resistance shown by most of these organisms, that is, the non-tuberculous mycobacteria species could be either intrinsic, attributed to a combination of the permeability barrier of the complex multilayer cell

Table 3. Antimicrobial susceptibility of non-tuberculous *Mycobacteria* species to methanol extract (mg/mL) of *A. leiocarpus* and *T. glaucescens*. Diameter (mm) of zone of inhibition ± SEM.

Plant sample	Concentrated of extract (mg/mL)	*M. fortuitum*	*M. smegmatis* ATCC 19420	*M. phlei*	*M. smegmatis*	*M. abscessus*
A. leiocarpus leaf	1	10± 0.0	11± 0.0	12± 0.5	11± 0.0	11± 0.5
	2	15± 0.5	14± 0.5	14± 0.5	17± 0.5	16± 0.0
	10	19± 0.5	18± 0.5	19± 0.0	19± 0.5	20± 0.0
	20	21± 0.0	22± 0.5	21± 0.5	21± 0.5	21± 0.0
A. leiocarpus stem bark	1	16± 0.0	11± 0.0	13± 0.5	13± 0.5	15± 0.0
	2	18± 0.0	15± 0.5	16± 0.5	16± 0.5	17± 0.0
	10	21± 0.5	22± 0.0	21± 0.5	21± 0.0	21± 0.0
	20	25± 0.5	24± 0.5	24± 0.5	23± 0.0	23± 0.5
A. leiocarpus root	1	13± 0.0	11± 0.0	10± 0.5	12± 0.0	12± 0.0
	2	15± 0.5	15± 0.5	15± 0.0	16± 0.5	15± 0.5
	10	20± 0.0	18± 0.5	22± 0.5	21± 0.0	20± 0.0
	20	22± 0.0	21± 0.0	24± 0.0	23± 0.5	24± 0.0
T. glaucescens root	1	13± 0.0	10± 0.0	15± 0.0	15± 0.0	13± 0.5
	2	16± 0.5	16± 0.0	20± 0.0	18± 0.0	18± 0.5
	10	21± 0.5	20± 0.5	22± 0.5	20± 0.5	20± 0.5
	20	23± 0.0	22± 0.0	25± 0.0	25± 0.0	22± 0.0
Rifampicin	20 µg/mL	19± 0.0	16± 0.5	17± 0.0	15± 0.0	16± 0.0
	40 µg/mL	21± 0.5	21± 0.0	23± 0.5	21± 0.5	21± 0.5
MeOH	40%	-	-	-	-	-

Diameter of cork borer = 8 mm, - = no zone of inhibition.

Table 4. Antimicrobial susceptibility of non-tuberculous *Mycobacteria* species to aqueous extract of *A. leiocarpus* and *T. glaucescens*. Diameter (mm) of zone of inhibition ± SEM.

Plant sample	Concentrated of extract (mg/mL)	*M. fortuitum*	*M. smegmatis* ATCC 19420	*M. phlei*	*M. smegmatis*	*M. abscessus*
A. leiocarpus leaf	1	10± 0.0	11± 0.0	10± 0.0	10± 0.0	10± 0.0
	2	14± 0.5	14± 0.5	14± 0.5	14± 0.0	15± 0.0
	10	18± 0.0	19± 0.5	18± 0.0	18± 0.0	19± 0.5
	20	21± 0.0	21± 0.5	20± 0.5	20± 0.0	21± 0.5
A. leiocarpus stem bark	1	10± 0.0	10± 0.0	10± 0.0	10± 0.0	10± 0.0
	2	12± 0.5	13± 0.5	14± 0.5	13± 0.5	14± 0.5
	10	18± 0.5	18± 0.5	17± 0.5	18± 0.5	19± 0.0
	20	21± 0.5	21± 0.5	20± 0.5	20± 0.0	21± 0.0
A. leiocarpus root	1	10± 0.0	10± 0.0	10± 0.0	10± 0.0	10± 0.0
	2	13± 0.0	14± 0.0	13± 0.5	13± 0.5	14± 0.0
	10	17± 0.5	17± 0.0	18± 0.0	18± 0.0	18± 0.0
	20	21± 0.5	21± 0.0	21± 0.5	21± 0.0	20± 0.5
T. glaucescens root	1	11± 0.5	10± 0.0	10± 0.5	10± 0.0	10± 0.5
	2	14± 0.0	13±0.5	13±0.5	13±0.5	14±0.0
	10	18± 0.0	18± 0.0	17± 0.5	18± 0.5	17± 0.5
	20	21± 0.0	22± 0.0	21± 0.0	21± 0.0	21± 0.5
Rifampicin	20 µg/mL	19± 0.0	16± 0.5	17± 0.0	15± 0.0	16± 0.0
	40 µg/mL	21± 0.5	21± 0.0	23± 0.5	21± 0.5	21± 0.5
MeOH	40%	-	-	-	-	-

Diameter of cork borer = 8 mm, - = no zone of inhibition.

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of aqueous extracts of *A. leiocarpus* and *T. glaucescens* on non-tuberculous *Mycobacteria* species.

Plant Sample	*Anogeissus leiocarpus* leaf		*Anogeissus leiocarpus* stem bark		*Anogeissus leiocarpus* root		*Terminalia glaucescens* root		Rifampicin	
Organism	**MIC (mg/ml)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (µg/mL)**	**MBC (µg/mL)**
M. fortuitum	1.25	5	1.25	2.5	1.25	5	2.5	2.5	5	10
M. smegmatis ATCC 19420	0.625	2.5	0.625	2.5	0.31	5	0.625	1.25	20	20
M. phlei	0.625	2.5	0.625	2.5	0.31	10	0.625	1.25	10	20
M. smegmatis	0.625	2.5	0.625	5	0.31	10	0.625	5	10	20
M. abscessus	0.625	2.5	0.625	2.5	0.31	5	0.625	1.25	10	20

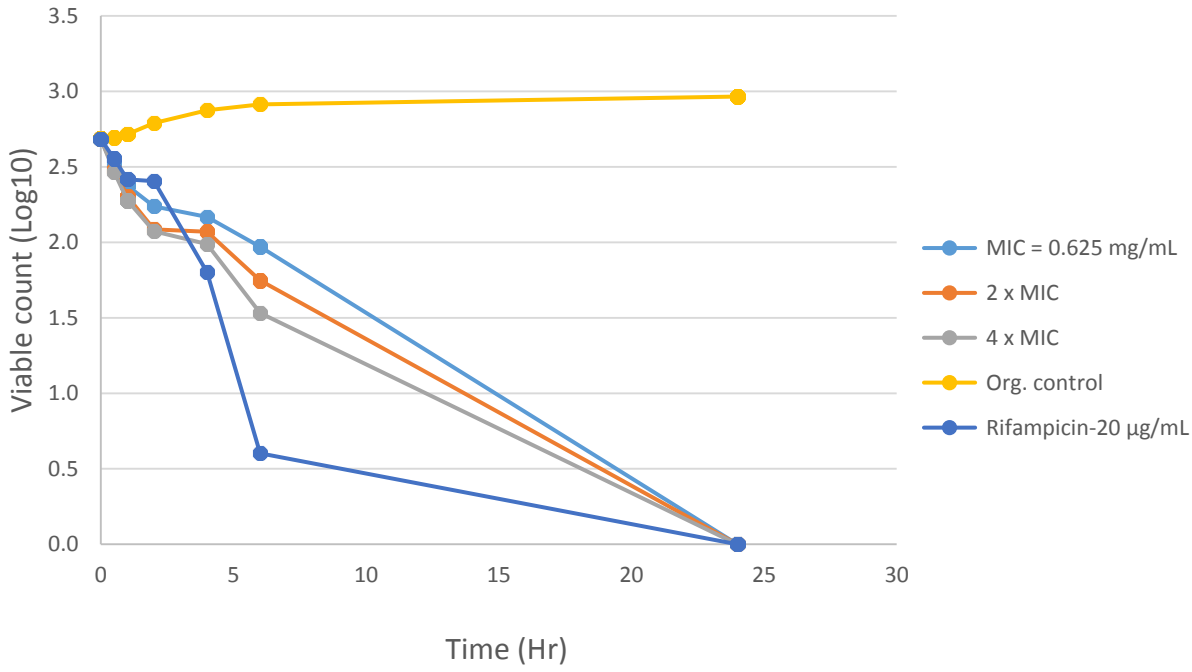

Figure 1. Plot of viable count (Log10) versus time (hour) of aqueous extract of *A. leiocarpus* (Leaf) on *M. smegmatis* ATCC 19420 showing the rate of kill of the organism at different concentrations of extract and exposure time to the extract.

envelope, drug export system, antibiotic targets with low affinity and enzymes that neutralize antibiotics in the cytoplasm; or acquired resistance through mutation. The cell envelope of mycobacteria species is usually waxy due to the presence of high lipid content accounting for about 60% of dry weight of the bacteria. This is considered a main factor contributing to their low permeability (Brennan and Nikaido, 1995) hence, the ability of the aqueous extracts to penetrate this barrier is notably significant. From our study, it can be deduced that the aqueous extracts of the test plants contain compounds that could be developed to elicit better anti-mycobacterial activity that will compare favourably with the drug/positive control which killed the organisms within the same contact time though at a lower concentration. This preliminary investigation agreed with the reports of Mann (2007) that the methanol extracts of *T. glaucescens* and *A. leiocarpus* inhibited the growth of *M. tuberculosis.*

Conclusion

The study investigated antibacterial activities of *A. leiocarpus* (DC.) (Guill. & Perr.) and *T. glaucescens* (Planch.) against nontuberculous mycobacteria species.

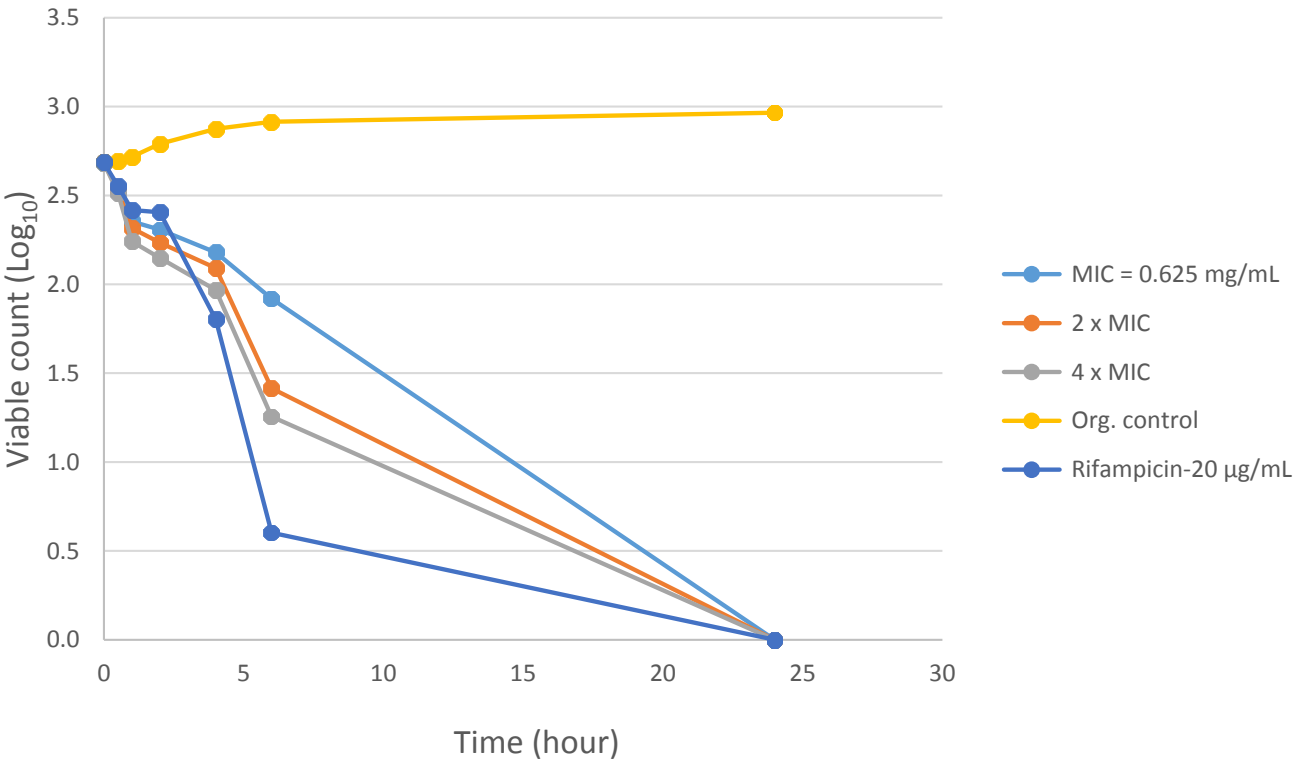

Figure 2. Plot of viable count (Log10) versus time (hour) of aqueous extract of *A. leiocarpus* (Stem bark) on *M. smegmatis* ATCC 19420 showing the rate of kill of the organism at different concentrations of extract and exposure time to the extract.

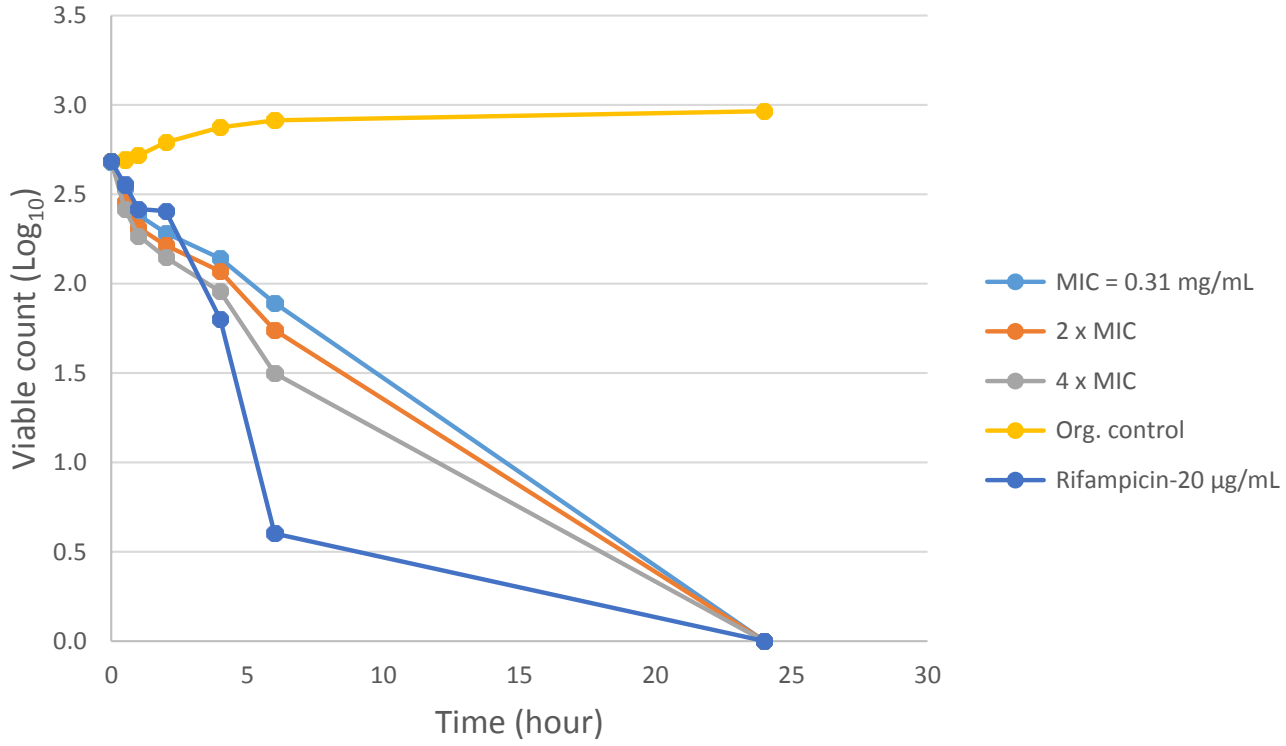

Figure 3. Plot of viable count (Log10) versus time (hour) of aqueous extract of *A. leiocarpus* (Root) on *M. smegmatis* ATCC 19420 showing the rate of kill of the organism at different concentrations of extract and exposure time to the extract.

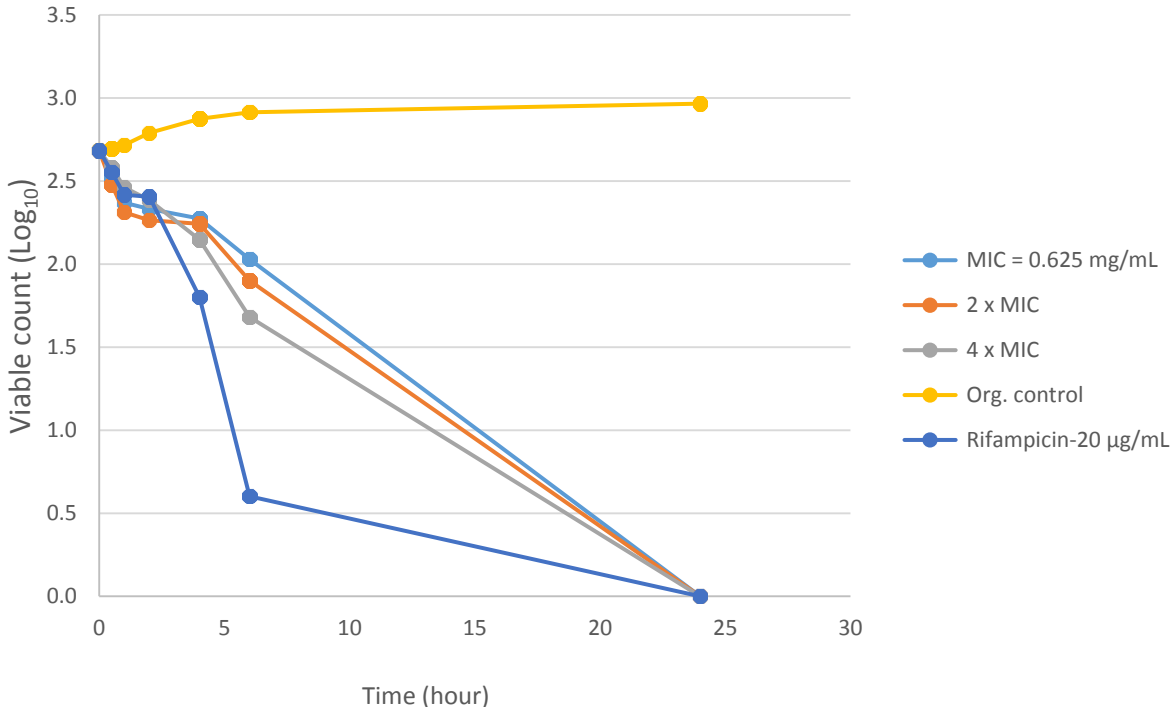

Figure 4. Plot of viable count (Log_{10}) versus time (hour) of aqueous extract of *T. glaucescens* on *M. smegmatis* ATCC 19420 showing the rate of kill of the organism at different concentrations of extract and exposure time to the extract.

These medicinal plants are used as medicines against infectious diseases including respiratory tract infections. The aqueous fractions of both plants had the highest activity on the test organisms suggesting the presence of bioactive components in the aqueous fractions. These findings support the use of these plants in traditional medicines where the plants are often used in the form of decoctions or are macerated in water for the treatment of various diseases including respiratory tract infections.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Fungal diversity and community structure in gut, mound and surrounding soil of fungus-cultivating termites

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The fungus-cultivating termites (Macrotermitinae) form part of diverse termite fauna in Africa, but information on their fungal symbionts is inadequate and poorly understood. In this study, the fungal communities and structure between termite gut, mound and surrounding soil were determined using the 454 pyrosequencing-based analysis of the internal transcribed spacer (ITS) gene sequences. Genomic DNA was extracted and purified from the guts of three termites (*Odontotermes* sp., *Macrotermes michaelseni* and *Microtermes* sp.), mound and surrounding soil samples for high-throughput sequencing. A total of 15,256 sequences were obtained and individual samples contained between 4 and 133 operational taxonomic units (OTUs). Termite gut had the least fungal diversity, dominated by members of the *Basidiomycota* (> 98%). More than 98% of the gut sequences were of the genus *Termitomyces*, while < 2% were related to the genera *Chaetomium, Fusarium, Eupenicillium, Cladosporium, Curreya* and *Phaeosphaeria* with between 95 and 98% pair-wise sequence identities. Members of *Ascomycota* (> 94%) were the most abundant in the mound and soil, but significantly differed (P value of 0.04; R value = 0.909) between the mound and soil environments. The results confirm that the genus *Termitomyces* exist in a tight association with their hosts and that *Termitomyces* species are scarcely present in the mound and soil. In addition, by altering soil properties; the fungus-cultivating termites modify the fungal community composition and structure in the mound and surrounding soil environments.

Key words: 454-pyrosequencing, microtermitinae, mutualism, tropical mycology.

INTRODUCTION

The diverse and numerous microorganisms in the soil perform key functions within the environment by participating in the cycling and flux of various nutrients, thereby influencing structure formation and sustenance of

soil properties (Holt and Lepage, 2000; Harry et al., 2001). Termites, a group of social insects consisting of over 2 600 species worldwide (Ahmed et al., 2011), are part of soil organisms that influence soil properties (Holt and Lepage, 2000; Harry et al., 2001; Manuwa, 2009; Muwawa et al., 2014). They are known as "soil engineers" as they have a great influence on the soil characteristics (Holt and Lepage, 2000), hence controlling diversity and activity of other soil organisms (Jones et al., 1997; Lavelle et al., 1997). Their influence on the soil microbial component is as a result of their major construction activities of complex galleries and mounds that result into soil heterogeneity in the tropical regions (De Bruyn and Conacher, 1990; Holt and Lepage, 2000).

The termite mound, thus, forms a specific habitat for soil microbiota since the physical and chemical properties are different from the surrounding soil (De Bruyn and Conacher, 1990; Holt and Lepage, 2000). The type of mound construction depends on the feeding habit of the termite species (Holt and Lepage, 2000). The fungus-growing termites build their mounds using soil and clay cemented by salivary secretions that make the mounds enriched with clay particles but impoverished in carbon (Harry et al., 2001). The nest-walls consist of organo-mineral aggregates, characterized by a low stability hence mineralize easily (Garnier-Sillam et al., 1988). They have a wider range of activity on the surrounding soil of 1 to 3 m in depth and within a range of a 2 to 8 m (Harry et al., 2001), which may influence the soil properties and fertility. The question is whether the fungus-feeding termites can be regarded as metabionts (Waid, 1999).

The Macrotermitinae comprises of the economically important termite species (Ahmed et al., 2011) that have been comprehensively studied (Mathew et al., 2012; Makonde et al., 2013; Otani et al., 2014, 2015; Muwawa et al., 2016). Previous studies have focused on the mutualistic symbiosis between *Termitomyces* sp. (Basidiomycota) and fungus-growing termites (Mohindra and Mukerji, 1982; Zoberi and Grace, 1990; Aanen et al., 2007, 2009; Osiemo et al., 2010; Nobre et al., 2010, 2011), parasitic fungi for termites (Traniello et al., 2002) and saprotrophic fungi such as *Xylaria* species that colonize termite nests (William, 1969; Moriya et al., 2005). Despite the termite activities influencing the microbial diversity and community structure, there is little information on comparative fungal community composition between termite gut, mound and corresponding soil environments. Therefore, in this study, we conducted a 454 pyrosequencing-based analysis of the ITS gene sequences to evaluate the gut fungal diversity associated with three fungus-cultivating termites. In addition, we evaluated on how, by altering soil properties; the fungus-cultivating termites modify the fungal community composition and structure in the mound and surrounding soil environments.

MATERIALS AND METHODS

Study sites and sampling

The samples were collected from Juja in Kiambu County, Kenya (latitude 1° 5′ 54.68″ N, longitude 37° 1′ 1.10″ W). The *Odontotermes* sp. (OTG1) [JQ247986] belonging to mound C, *Macrotermes michaelseni* (MTG4) [JQ247993] and *Microtermes* sp. (MIG7) [JQ247990] both colonizing mound D (~2 km far away from mound C) were sampled by excavating each mound to a depth of approximately 1.0 m and aseptically collecting the termites (n = 200 workers and 50 soldiers). Worker-caste termites were used in the experiments due to their foraging behavior. The identity of the termites was confirmed by sequencing the mitochondria cytochrome oxidase II gene in DNA extracted from the heads of soldiers (Austin et al., 2004) and comparing it to the sequences of previously identified specimens (Inward et al., 2007). In addition, soil samples (~40 g collected at ~5 cm depth) from termite mounds (OTN2 and MTN5) and surrounding soil samples (OTS3 and MTS6, collected at 3 m away from termite mounds C and D, respectively) were included in the analyses.

DNA extraction

DNA extraction was performed as described previously (Makonde et al., 2013). Briefly, the exterior surfaces of the termites were washed with 70% ethanol and then rinsed with sterile distilled water. The guts were aseptically removed with forceps. A total of 165 guts (~1 g) of the *Odontotermes* sp. (OTG1) and *M. michaelseni* (MTG4) and 198 guts (~1g) of *Microtermes* sp. (MIG7) were separately put into sterile micro tubes containing 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). They were then homogenized using a sterile glass rod. The corresponding homogenates were then transferred into sterile tubes and used for total DNA extraction. The soil samples were homogenized separately and debris were removed. Subsequently, soil samples (~4 g) were used for total microbial DNA extraction. Total DNA extraction for all samples was performed using MoBio PowerMax Soil DNA isolation kit (MoBio Laboratories, Inc. CA, USA) according to the manufacturer's protocol. DNA concentration was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA) as recommended by the manufacturer.

Amplification of internal transcribed spacer (ITS) gene region and sequencing

The fungal DNA was PCR amplified using a set of the universal ITS gene primers (the ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS4 [5'-TCCTCCGCTTATTGATATGC-3']) according to White et al.

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(1990). These fungal primer set were modified for 454 pyrosequencing by attaching an adaptor sequence, a key and a unique 12 Nucleotide MID for multiplexing purposes. Each PCR reaction (50 µL) contained forward and reverse primers (10 µM, each), dNTP's (10 mM each), Phusion GC buffer (Finzymes), Phusion high fidelity polymerase (0.5 U μL^{-1}) and 25 ng of template DNA. Amplifications occurred in an Eppendorf Mastercycler Thermal Cycler with the following program conditions: An initial heating at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min, after which a final extension step at 72°C for 5 min was performed. The amplification was confirmed using gel electrophoresis of 2 µl of the PCR product on a 1% TAE agarose gel (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, 1.5% (w/v) for 1 h at 100 V. Later three independent PCR products per sample were pooled in equal amounts, separated on a gel and extracted using the peqGOLD gel extraction kit (PeqLab Biotechnologie GmbH, Erlangen, Germany). Quantification of the PCR products was performed by using the Nanodrop (NanoDrop Technologies, USA) method and a Qubit fluorometer mbH, (Invitrogen GmbH Karlsruhe, Germany) as recommended by the manufacturer. Sequencing of the PCR amplicons was done at the Göttingen Genomics Laboratory using Roche GS-FLX 454 pyrosequencer (Roche, Mannheim, Germany) as recommended in the instructions of the manufacturer for amplicon sequencing.

Data analysis

Raw sequences were quality filtered according to Huse et al. (2007) using the QIIME release 1.9.0 software (Caporaso et al., 2010). Briefly, low quality sequences were removed from the analysis if they were less than 200 bp in length, contained ambiguous characters, did not contain the primer sequence or contained an uncorrectable barcode. The remaining sequences were assigned to samples based on the 12-nucleotide barcode. The denoised sequences were evaluated for potential chimeric sequences using UCHIME in the USEARCH package v.4.2.66 (Edgar, 2010). A sequence identity cutoff of 97% was used to pick OTUs from the quality filtered non-chimeric sequences. Representative OTUs were picked using the de novo OUT clustering (Rideout et al., 2014) with standard UCLUST method using the default settings as implemented in QIIME at 97% similarity level. OTU alignment was performed using the python implementation of the NAS algorithm, PyNAST (Caporaso et al., 2010). Taxonomy was assigned to representative sequences from each cluster using BLASTn against the SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast et al., 2013) at dissimilarity levels of 3, 5 and 10%. Rarefied datasets were generated with the multiple_rarefaction function in QIIME in order to remove sample heterogeneity before diversity assessment. Rarefaction curves and diversity indices were calculated and plotted for each sample using QIIME (Caporaso et al., 2010). To determine the amount of dissimilarity (distance) between any pair of bacterial communities, we used the UniFrac metric (Lozupone and Knight, 2005; Lozupone et al., 2007) that incorporates the degree of divergence in the phylogenetic tree of OTUs into Principal coordinates analysis (PCoA). A relatively small UniFrac distance implies that two communities are compositionally similar, harboring lineages sharing a common evolutionary history. In unweighted UniFrac, only the presence or absence of lineages is considered. We used the analysis of similarities (ANOSIM) (Clarke, 1993; Fierer et al., 2010) through 1000 to test for differences in community composition among the groups of samples. Additionally, the relative abundance of the genera was used in hierarchical clustering using the pearson correlation distance metric implemented in MultiExperimentViewer version 4.9.0 (MeV 4.9.0). Fungal communities across the analyzed samples were compared based on the relative abundances of some selected fungal genera, using principal component analysis (PCA) as implemented in R (R Core Team, 2012). All pyrosequencing-derived ITS gene sequences datasets were deposited in the GenBank under accession number SRP019764.

RESULTS

Distribution of Fungal phyla across the samples

The overall reads for the fungal samples were 18,294. After quality filtering and chimera check 15,256, the resulting sequences (≥ 200 bp) were clustered into 287 OTUs (Table 1) at 3% sequence divergence. Taxonomic assignment of the resulting sequences against the SILVA database showed ≥ 2 known phyla, but the major ones (> 90% of the analyzed sequences) were *Ascomycota* and *Basidiomycota* (Figure 1).

Fungal community composition across samples

The abundance of fungal composition at the phylum level differed across the samples (Figure 1; Table 1). Members of the phylum *Basidiomycota* were the most abundant (>98% of the analyzed sequences) in the gut samples [MIG7, MTG4 and OTG1] compared to those of mound [sample OTN2] and soil environments [samples OTS3 andMTS6], which were predominated by members of the phylum *Ascomycota* [>94% of the analyzed sequences] (Figure 1). There were no sequences for sample MTN5 due to some sequencing errors. At the class level, members affiliated with *Agaricomycetes* were the most abundant (>98%) in the gut samples [MIG7, MTG4 and OTG1], but least in the mound (OTN2) and soil (OTS3 and MTS6) samples (Table 2). Members of *Sordariomycetes* and *Eurotiomycetes* were the most abundant in the mound (89%) and soil (54-68%) samples, respectively. Other classes such as *Dothideomycetes* (4.5%), *Eurotiomycetes* (3.6%) and uncultured a*scomycete* (1.1%) were relatively abundant in the mound, while classes such as *Sordariomycetes* (≥12%), *Dothideomycetes* and *Orbiliomycetes* (>5%) were relatively abundant in the soil (Table 2).

At the order level, the relative abundances of the fungal communities in the samples were different. The order Agaricomycetidae was the most abundant group in the termite gut. Notably, the mound was dominated by the order Hypocreomycetidae while the soil was predominated by members of the order Eurotiomycetidae (Figure 2). Other orders such as Dothideomycetidae, Pleosporomycetidae, Chaetothyriomycetidae, Sordariomycetidae, Xylariomycetidae and Orbiliales were detected at varying relative abundances (1 to 22% of the analyzed sequences) in some samples (Figure 2).

At the genus level, the most abundant genus in the gut

Table 1. Number of sequences, observed OTUs, the estimated richness and diversity indices at 3% dissimilarity threshold.

Sample ID	Sample description	Reads before QT	Reads after QT	OTUs	Phyla	Classes	Richness and diversity indices				
							Chao1 index	ACE	Simpson (1/D)	Shannon	Fisher_alpha
OTG1	*Odontotermes* sp. gut homogenate	1,569	1,421	5	3	3	6.5	11	0.002	0.01	0.54
OTN2	Soil from mound C of *Odontotermes* sp.	2,369	2000	53	5	12	59	56.9	0.61	2.4	8.2
OTS3	Soil collected 3 m away from mound C	3,227	2,505	83	4	11	92.3	87.6	0.82	3.7	13.3
MIG7	*Microtermes* sp. gut homogenate	2,614	2373	4	2	2	5	8.1	0.003	0.02	0.42
MTG4	*M. michaelseni* gut homogenate	2,000	1,815	9	2	3	10	10	0.03	0.2	1.2
MTN5	Soil from mound D of *M. michaelseni*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MTS6	Soil collected 3 m away from mound D	6,515	5142	133	5	16	144	145.5	0.79	3.4	23.4
		18, 294	**15, 256**								

QT', Quality trimming; 'OTUs', operational taxonomic units; 'ND', not determined.

samples was *Termitomyces* (>98%), but was detected at low relative abundance (< 0.4%) in the mound and soil samples (Table 3). Notably, fungal species associated with *Eupenicillium limosum*, *Monilinia fructicola* and *Fusarium oxysporum* (with 96 to 98% sequence identities) were detected in the gut of *Odontotermes* sp. (sample OTG1). These fungi, however, constituted about 0.1% of the analyzed sequences. Likewise, in the gut of *Microtermes* sp. (sample MIG7), about 0.1% of the sequences were related to *Trichocoma paradoxa* and *Cladosporium* sp. CF-25 (with 96 to 98% sequence identities). The gut of *M. michaelseni* (sample MTG4), had about 1.3% of the analyzed sequences affiliated with *Chaetomium globosum, Myrothecium* sp. J3, *Monodictys castaneae, Fusarium oxysporum, Penicillium purpurogenum, Cladosporium* sp. CF-25, *Phaeosphaeria avenaria* and *Curreya pityophila* [with 96 to 99% sequence identities] (Table 3).

The genus *Fusarium* [17%] and particularly the genus *Hypocrea* [59%] were the most abundant genera in the mound (Table 3), but the soil samples were predominated by the following genera; *Aspergillus* [45%], *Eupenicillium* [39%] and *Xylaria* [19%]. In the mound (OTN2), most of the fungal species were affiliated with *H. koningii*, *Fusarium* sp. CPCC 1400009 and *C. globosum* (with 97 to 99% sequence identities) while in the surrounding soil, the fungal species were between 97 and 98% affiliated with *E. limosum*, *A. fumigatus*, *Xylaria hypoxylon* and *Hypocrea koningii* (Table 3).

Fungal diversity and richness

Fungal diversity and richness for the analyzed sequences for each sample (Table 1) were evaluated by rarefaction (Figure not shown). At 3% sequence divergence, some rarefaction curves did not reached saturation, indicating that the surveying efforts did not fully cover the extent of taxonomic diversity at this genetic distance, but a substantial fraction of the fungal diversity within individual samples was evaluated. The diversity measures showed that MTS6 had the most genus-level taxa (133; Table 1) and MIG7 the least (4; Table 1), that MTS6 was richest (Chao 1 index), while MIG7 was poorest. There was variation in community composition as indicated by the Simpson (1/D) and Shannon indices (Table 1).

Comparison of the individual samples using unweighted UniFrac PCoA (Figure 3) showed a distinct clustering by environment, but the *p*-value of 0.04 and R value of 0.909 indicated that at an alpha of 0.05; the grouping of samples is relative strong implying that there is dissimilarity between the groups. For instance, the gut samples (MIG7, OTG1 and MTG4) did not cluster together and with those of mound and soil (Figure 3 and 4), indicating dissimilarity in the fungal communities. Likewise, samples OTS3, MTS6 and OTN2 did not cluster together, indicating that each individual sample had almost different fungal communities. Notably, the mound sample (OTN2) did not cluster with its corresponding soil sample (OTS3), implying that the mound fungal community composition was different from that of its surrounding soil (Figures 3 and 4).

The PCA shows that the fungal communities within the termite gut are mainly impacted by the genus *Termitomyces* while those of the mound

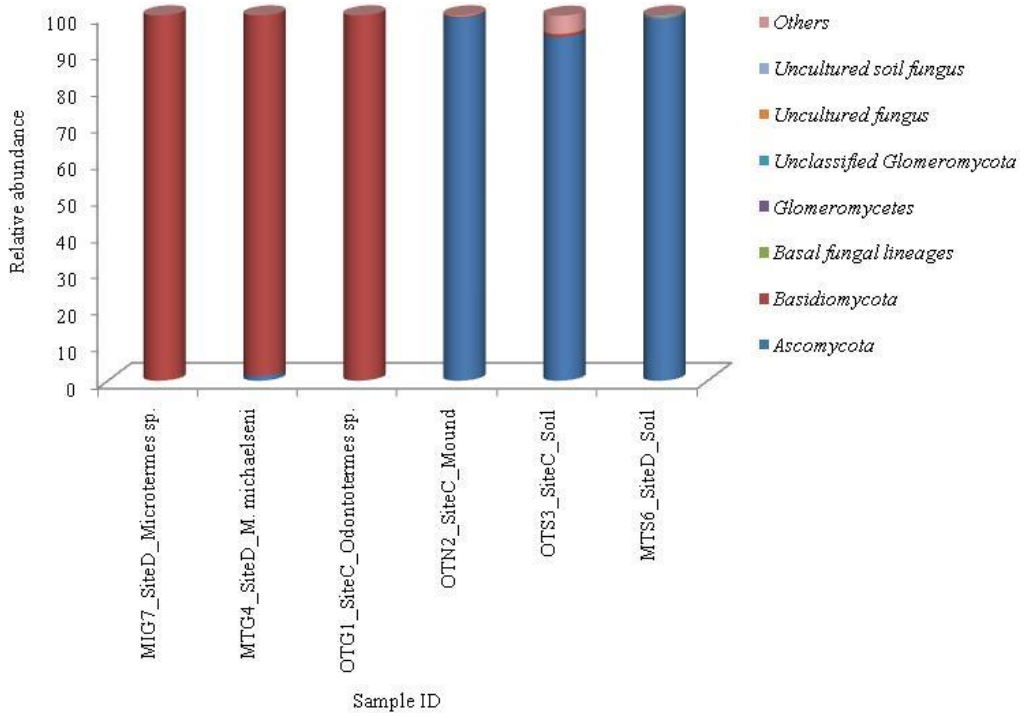

Figure 1. Relative abundances (%) of fungal phyla in the samples. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, soil from mound C of *Odontotermes* sp.; MTS6, soil collected 3 m away from mound D; OTS3, soil collected 3 m away from mound C. Phylogenetic groups accounting for < 0.4% of the analyzed sequences were included in the artificial group 'others'.

Table 2. Distribution of the fungal sequences into class level after quality trimming.

Class	Termite gut			Mound	Soil	
	MIG7	MTG4	OTG1	OTN2	OTS3	MTS6
Dothideomycetes	0.1	0.9	0	4.5	7	5.2
Eurotiomycetes	0	0	0.1	3.6	67.2	54.9
Lecanoromycetes	0	0	0	0	0.3	0.3
Lichinomycetes	0	0	0	0	0	0.2
Orbiliomycetes	0	0	0	0	5.8	0
Sordariomycetes	0	0.4	0	89	12	36.4
Taphrinomycetes	0	0	0	0.6	0	0.2
Uncultured rhizosphere ascomycete	0	0	0	1.1	0	0.3
Coniosporium	0	0	0	0	0.4	0
Humicola	0	0	0	0	0.8	0
Lecophagus	0	0	0	0	0	0.3
Phoma	0	0	0	0	0.6	0.6
Pseudosigmoidea	0	0	0	0.2	0	0
Agaricomycetes	99.9	98.7	99.9	0.3	0.8	0.2
uncultured Basidiomycota	0	0	0	0.1	0	0
unclassified Mucoromycotina	0	0	0	0	0	0.1
Other	0	0	0	1	5	1.1

MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, soil from mound C of *Odontotermes* sp.; MTS6, soil collected 3 m away from mound D; OTS3, soil collected 3 m away from mound C. Phylogenetic groups accounting for < 0.1% of the analyzed sequences were included in the artificial group 'others'

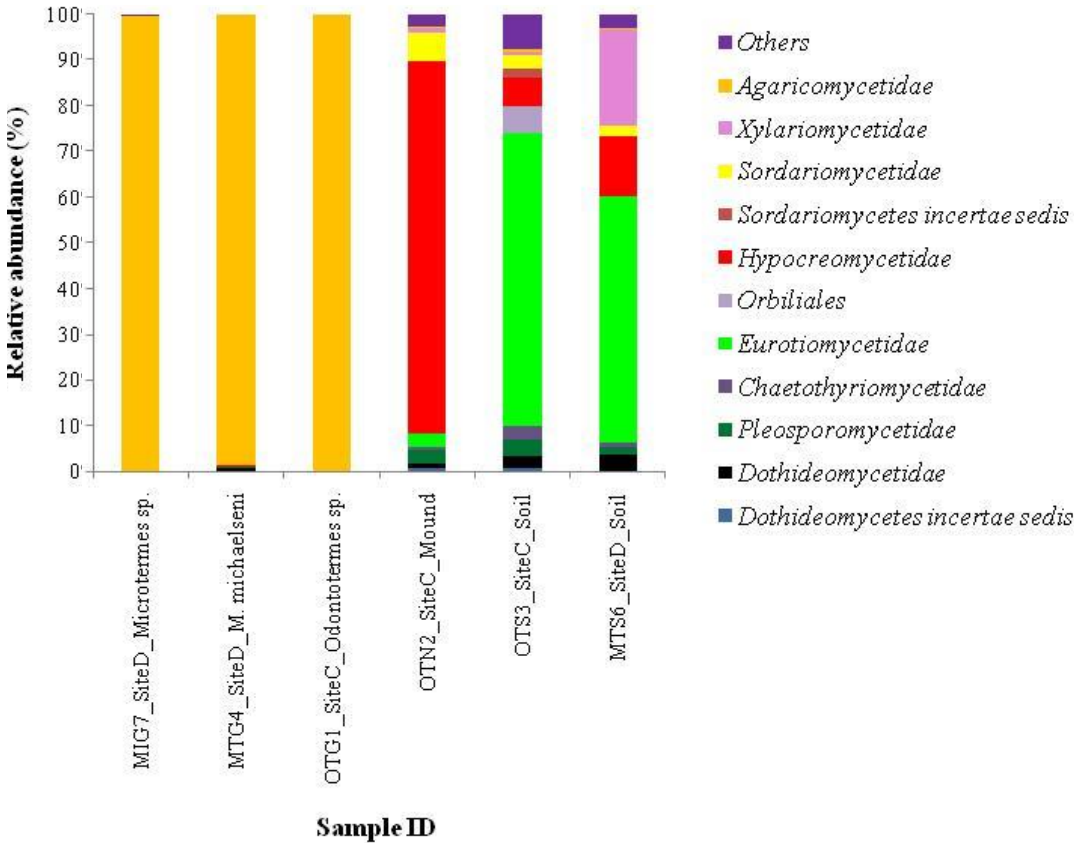

Figure 2. Relative abundances of the orders in the domain Eukaryota. Unknown Phylogenetic groups are included in the artificial group 'others'. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

are impacted by the genera *Fusarium* and *Hypocrea* (Figure 5). The surrounding soil is impacted by the genera *Xylaria, Aspergillus* and *Eupenicillium*. Their relative abundances varied across the samples (Table 3). On one hand, *Termitomyces* species were the most dominant fungal species in the gut of three fungus cultivating termites (*M. michaelseni, Odontotermes* and *Microtermes* species), while members of the genera *Fusarium* and *Hypocrea* were more dominant in the mound. On the other hand, members of the genera *Xylaria, Aspergillus* and *Eupenicillium* were more predominant in savannah soil (Table 3 and Figure 4).

DISCUSSION

Defining the number of fungi on the planet has always been an area of debate (Hawksworth, 2001), but has recently gained prominence in scientific literature. This has provided the foundation for studies aimed at obtaining a better understanding of fungal biodiversity worldwide. Termites and their mounds harbor diverse organisms including fungi. There are two aspects of fungal diversity on termite mounds, namely, the presence of *Termitomyces* versus other fungi such as *Xylaria/ or Pseudoxylaria* species (Moriya et al., 2005; Okane and Nakagiri, 2007; Ju and Hsieh, 2007; Guedegbe et al., 2009; Visser et al., 2009; 2012) and the diversity within *Termitomyces* species.

Our study compared the fungal diversity and community structure in the termite gut, mound and surrounding soil. The results of our study revealed two major fungal phyla; *Ascomycota* and *Basidiomycota* whose members' distribution differed significantly across the samples. The phylum *Basidiomycota* was the most abundant in the termite gut while the phylum *Ascomycota* dominated in the mound and surrounding soil. Furthermore, members of *Ascomycota* differed significantly between the mound and surrounding soil (Table 3 and Figures 2 and 4). The discrepancy of fungal composition between the mound and soil may emanate from the construction activities of the termites. Such activities can chemically modify the

Table 3. Relative abundances (%) of the genera in the domain eukaryota.

Phylum	Genus affiliation	Termite gut			Mound	Soil		%ID
		MIG7	MTG4	OTG1	OTN2	MTS6	OTS3	
Basidiomycota	*Termitomyces* sp. ZA164	99.9	0	0	0	0.1	0	98
Basidiomycota	*Termitomyces* sp. ZA164	0	0	99.9	0.3	0	0.4	98
Basidiomycota	*Termitomyces* sp. ZA164	0	98.6	0	0	0	0	96
Ascomycota	*Xylaria hypoxylon*	0	0	0	0.7	19.6	0.8	98
Ascomycota	*Chaetomium globosum*	0	0.04	0	10.1	1.5	2.6	99
Ascomycota	*Ceratostomella pyrenaica*	0	0	0	0	0	0.5	96
Ascomycota	*Papulosa amerospora*	0	0	0	0	0.1	1.6	96
Ascomycota	*Papulosa amerospora*	0	0	0	0	0	0.35	96
Ascomycota	*Papulosa amerospora*	0	0	0	0	0	0.3	95
Ascomycota	*Fusarium oxysporum*	0	0	0.02	0.7	1.2	0.5	96
Ascomycota	*Fusarium* sp. CPCC 1400009	0	0	0	15.2	0.8	0	97
Ascomycota	*Fusarium* sp. 18014	0	0	0	0.8	0.2	0	96
Ascomycota	*Hypocrea koningii*	0	0	0	59.7	12.5	5.7	98
Ascomycota	*Helicoon fuscosporum*	0	0	0	0	0	6.5	96
Ascomycota	*Aspergillus fumigates*	0	0	0	0.12	42.3	1.4	99
Ascomycota	*Sagenomella humicola*	0	0	0	0.2	0.8	0.5	97
Ascomycota	*Sagenomella humicola*	0	0	0	0	0.5	0	95
Ascomycota	*Sagenomella humicola*	0	0	0	0	2.9	0	95
Ascomycota	*Aspergillus* sp. LQ21	0	0	0	0	1.5	0.5	97
Ascomycota	*Aspergillus clavatus* NRRL 1	0	0	0	0	0.4	0.5	97
Ascomycota	*Eupenicillium limosum*	0	0	0.02	2.4	1.1	39.4	97
Ascomycota	*Phaeosphaeria avenaria f. sp. Avenaria*	0	0.2	0	1.1	0.1	0.2	98
Ascomycota	*Curreya pityophila*	0	0.4	0	1	1.9	4.4	98
Ascomycota	*Curreya pityophila*	0	0	0	0.6	0.2	0.1	95
Ascomycota	*Cladosporium* sp. CF-25	0.01	0.6	0	0.5	1.3	1.6	98
Ascomycota	*Leptoxyphium fumago*	0	0	0	0.8	1.7	0.2	99
Others		0.09	0.08	0.06	5.68	9.3	31.95	

Phylogenetic groups that are (≤ 0.3%) in all samples are included in the artificial group 'others'. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

organic matter in the mound (Holt and Lepage, 2000; Harry et al., 2001), hence creating ecological microniches suitable for more specialized fungi.

The most abundant fungal genus in the termite gut was *Termitomyces*, which was represented by over 98% of the analyzed sequences in each gut sample (Table 3). Notably, the gut *Termitomyces* symbiont differed in the host. For instance, an interesting scenario was noted in mound D, which was inhabited by two different termite species. Each termite species (*M. michaelseni* vs. *Microtermes* sp.) cultivated its own *Termitomyces* strain (Table 3). Since the *Macrotermes* and *Microtermes* termites colonized the lower and upper parts, respectively, the likelihood of horizontal transfer of the fungus should have been high as suggested previously (Makonde et al., 2013). But this was not the case; thus, the affected host-*Termitomyces* relationships are likely to be too specialized to allow host switching. Literature indicates that some termite genera cultivate a restrictive set of fungal symbionts (Aanen et al., 2007; Osiemo et al., 2010). Nonetheless, it remains to be addressed how the termites exclusively select the right *Termitomyces* symbiont for their colony. The mound and soil samples (OTS3, MTS6 and OTN2) did not cluster together, indicating that each individual sample had almost different fungal communities.

Besides, the 454-pyrosequencing approach used in this study revealed other minor fungal species, which were previously often undetected by the traditional Sanger sequencing in the termite gut (Mathew et al., 2012; Makonde et al., 2013). This is because the use of the clone-based approach for microbial analyses in the previously mentioned studies could have been limited by PCR errors and bias in selecting representative clones

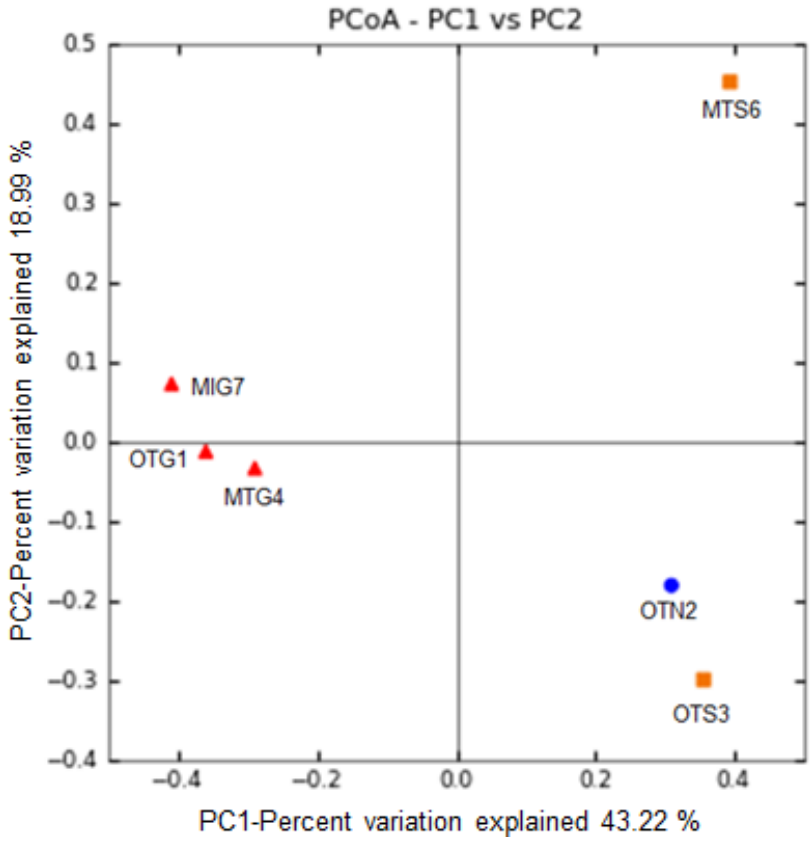

Figure 3. PCoA plots showing the degree of similarity of bacterial communities on termite guts, mounds and soil samples. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

for sequencing. In this current study, fungal species affiliated with *E. limosum, M. fructicola* and *F. oxysporum* were detected in the gut of *Odontotermes* species (Table 3). This, however, constituted about 0.1% of the effective sequences just like in the gut of *Microtermes* species, where 0.1% of the sequences were associated with *T. paradoxa* and *Cladosporium* sp. CF-25 (with 96 to 98% sequence identities). In the gut of *M. michaelseni*, about 1.4% of the sequences were related to *Chaetomium globosum, Myrothecium* sp. J3, *M. castaneae, F. oxysporum, P. purpurogenum, Cladosporium* sp. CF-25, *P. avenaria* and *C. pityophila* (with 96 to 99% sequence identities). Elsewhere, Mathew et al. (2012) isolated yeasts closely related to *Debaryomyces hansenii, Pichia guilliermondii* and *Candida inconspicua* from the comb material and gut of *Odontotermes formosanus* using adapted cultivation techniques. However, these fungi/yeasts were detected insignificantly in quantitative terms, and it is unclear which role they play. In contrast to our findings, some previous studies conducted using clone-based approach (Mathew et al., 2012) could not identify fungal genera in some fungus-cultivating termites' guts other than *Termitomyces*.

Though the results of this study do not support the physiological roles of the symbiotic fungi detected, several researchers have proposed roles associated with symbiotic fungi (*Termitomyces* species) in termites. For example, provision of glycosyl hydrolases (Martin and Martin, 1978), enrichment of nitrogen, which is advantageous as the dead plant material consumed by termites, is poor in nitrogen (Collins, 1983), and lignin degradation, which subsequently allow for cellulose digestion (Hyodo et al., 2000). Nonetheless, the significance of each role differs in value among termite species (Rouland-Lefévre, 2000; Hyodo et al., 2003). Hyodo et al. (2003) suggested that the important role of symbiotic fungi in *Macrotermes* species is to degrade lignin, hence allowing for efficient digestion of cellulose, whereas for *Odontotermes* species, *Hypotermes makhamensis* and *Phidiana militaris*, it is to serve a

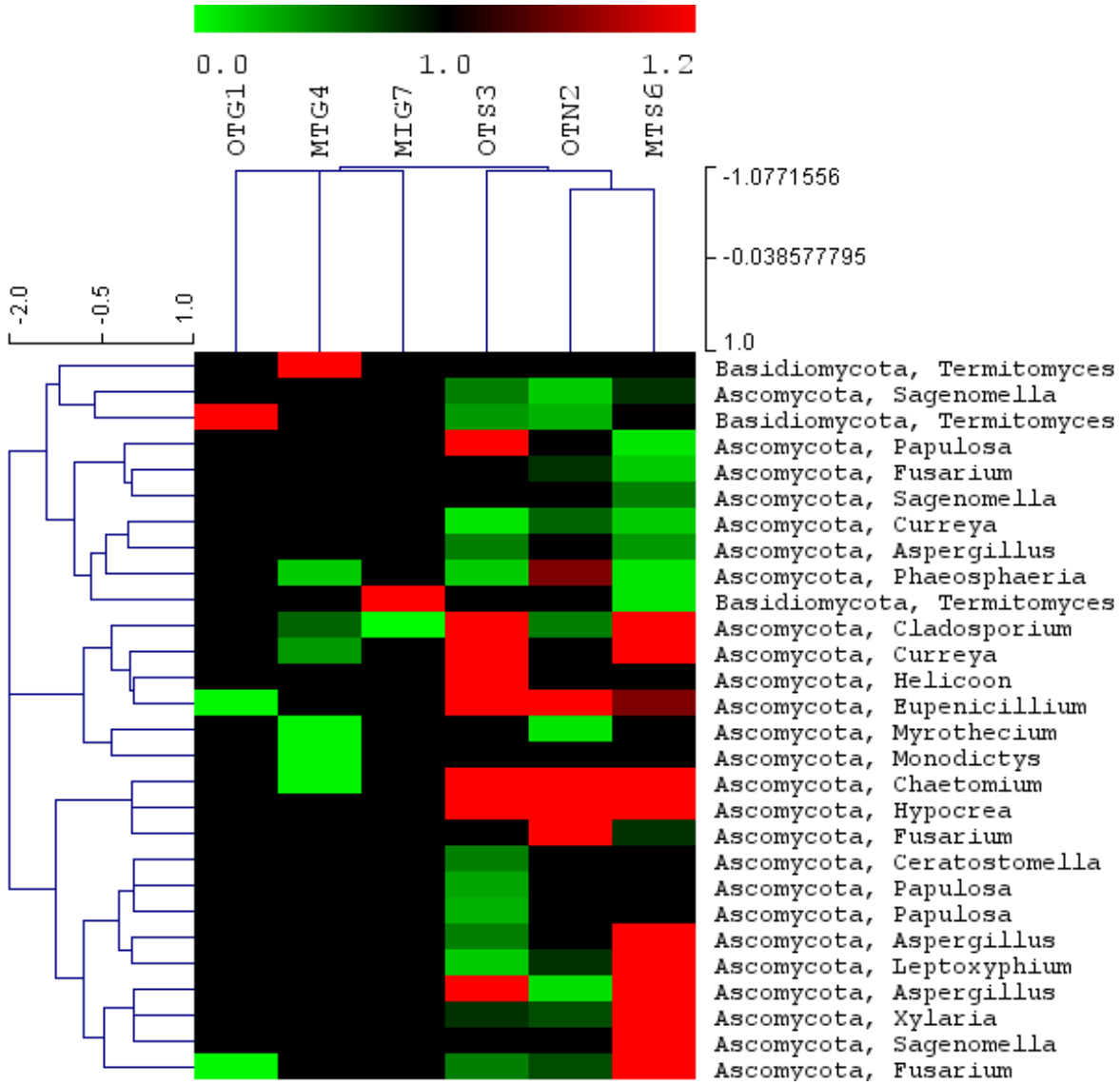

Figure 4. Heatmap shows hierarchical clustering of taxa (relative abundance > 0.1% of the analyzed sequences). The scale bar represents color saturation gradient based on the relative abundances of the fungal genera. The dendrogram at the top shows the weighted Euclidean distance analysis of community similarity. Classification is presented at the genus and phylum levels. OTG1, *Odontotermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; MIG7, *Microtermes* sp. gut homogenate; OTN2, soil from mound C of *Odontotermes* sp.; MTS6, soil collected 3 m away from mound D; OTS3, soil collected 3 m away from mound C.

nutritional role. However, it is still unclear whether the different roles of such fungi are directly dependent on termite taxonomy or variation in plant biomass used to make fungus comb (Hyodo et al., 2003).

The genus *Fusarium* and particularly the genus *Hypocrea* were the most abundant genera in the mound compared to the soil, which was dominated by the genus *Aspergillus* among others (*Eupenicillium*, *Xylaria* and *Hypocrea*). On one hand, the transformed soil properties in the mound might have favored the proliferation of particular fungi; especially those related to the genera *Fusarium* and *Hypocrea.* On the other hand, it might have limited the growth of other genera such as *Aspergillus, Xylaria* and *Eupenicillium,* which were mostly favored by conditions in the surrounding soil. Thus, the soil harbored a higher diversity of fungi most of which were different from those of the mound and gut. The differences in fungal communities between the mound and surrounding soil may partly be attributed by influence of the fungus-cultivating termites on the soil properties, which

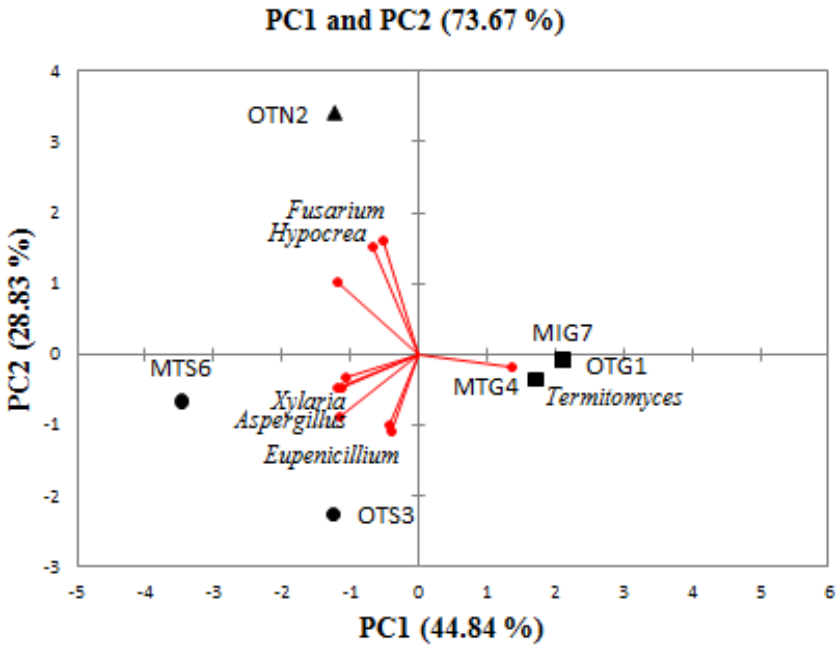

Figure 5. Principal component analysis (PCA) of fungal communities based on the relative abundances of the fungal genera. The vectors indicate the direction and impact of each detected fungal genera on the overall variance. Sample types are marked by the black rectangles, triangles and circles, respectively. MIG7, *Microtermes* sp. gut homogenate; MTG4, *M. michaelseni* gut homogenate; OTG1, *Odontotermes* sp. gut homogenate; OTN2, Soil from mound C of *Odontotermes* sp.; OTS3, Soil collected 3 m away from mound C; MTS6, Soil collected 3 m away from mound D.

consequently modify the diversity and composition of fungal communities. Previously, Chen and Cairney (2002) demonstrated that perturbation of Australian forest soils affected the fungal composition while Landeweert et al. (2003) observed difference in basidiomycete community between the organic and mineral horizons. The activities of other biota can modify soil properties and might be the same factor affecting fungal diversity within the same region and vegetation type (McLean and Parkinson, 2000).

The current findings underline the difference on fungal community composition between the gut, mound and surrounding soil. The heterogeneity of the organic matter, occurrence of fungal inhibitors (Chen and Cairney 2002; Lamberty et al., 2001) and the creation of new substrates/or reduced access for fungi in such clay organic complexes could favor some specialized fungal species (Roose-Amsaleg et al., 2004). As a result, fungus-cultivating termites could be regarded, according to Waid (1999), as true metabionts since they create special micro-environments that support specific organisms such as fungi that may adapt, evolve and hence diversify. Such a scenario has been observed on soil-feeding termites; which by modifying the environment drastically affected the soil ascomycete community structure (Roose-Amsaleg et al., 2004).

Soil fungi mediate many biochemical interactions (Bridge and Spooner, 2001) including a variety of associations with plants as pathogens (e.g *Fusarium* spp.), while other genera such as *Aspergillus*, *Penicillium* and *Xylaria* could be saprophytes, necrophilia and even coprophile. Several species of fungi associated with *Reticulitermes flavipes* have been isolated (Zoberi and Grace, 1990), many of which were common saprophytic soil organisms (Barnett and Hunter, 1972). *Mucor mucedo* (L.) Fr. and *Aspergillus niger* Van Tieg. (Steinhaus, 1949) are known to be facultative insect pathogens while *Mucor hiemalis* Weh., was reported as a pathogen of bees. It is worthy of note that the multi-species fungal interactions such as competitive or parasitic interactions (Zoberi and Grace, 1990) among fungi promote termite survival as supported by a number of species associated with living termites.

Conclusion

The findings of this study have demonstrated that members of the genus *Termitomyces* exist in a tight association with their hosts (Rouland-Lefevre, 2000),

hence *Termitomyces* species are scarcely present in the mound and soil. In addition, by altering the habitat, fungus-cultivating termites create microecological niches suitable for some specialized soil fungal species. The use of 454-pyrosequencing has demonstrated the existence of other minor fungal genera in the termite guts other than *Termitomyces,* which is the dominant fungus. This demonstrates that the mutualistic association of the *Termitomyces* with termites is important for their survival. Therefore, further studies should be focused on host-symbiont specificity and physiological roles of the host symbionts for further exploitation particularly in the field of biotechnology.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

In silico analysis of Chikungunya virus (CHIKV), a mosquito-borne alphavirus

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Chikungunya virus (CHIKV) is a mosquito-borne alpha virus, which belongs to the family, *Togaviridae*. This virus is known to cause an acute onset of high fever, severe arthralgia and rash, and is usually accompanied by headache and severe joint pain. The present study aimed to construct an updated phylogenetic tree of currently published data and perform a phylogeographic analysis of Chikungunya virus obtained during different outbreak in the last five years after the re- emerging of chikungunya virus to get further insight into the epidemiology and transmission of CHIKV. In this study, twenty two sequences from the E1envelope glycoprotein gene were aligned using ClustalW software program. A phylogenetic tree was constructed by using MEGA 5 software version 6, to determine the phylogenetic relationships of CHIKV during different outbreak recently in Yemen, Italy, Philippines, India and Africa. An updated phylogenetic tree was constructed, the results obtained suggested that CHIKV strains isolated recently in the Eastern Mediterranean Region share high similarity with chikungunya virus isolated in Tanzania in 1953.

Key words: Chikungunya fever, epidemiology, outbreaks, phylogenetic tree.

INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus, that belongs to the family of *Togaviridae* (Schuffenecker et al., 2006), this virus is known to cause an acute onset of high fever, severe arthralgia and rash, and is usually accompanied by headache and severe joint pain (McGill, 1995; Adebajo, 1996; Mourya et al., 2006; Ligon, 2006; Yazdani et al., 2007; Leparc-Goffart et al., 2014). CHIKV is principally transmitted to humans via the bite of an infected anthropophilic vector *Aedes aegypti* and *Aedes albopictus* (Centers for Disease Control and Prevention, 2011).

CHIKV is a spherical, enveloped, positive-strand RNA virus (Higashi et al., 1967; Simizu et al., 1984) with a genome of 12 kb, CHIKV genome contains two ORFs, which encodes for structural and non-structural polyproteins (Khan et al., 2002). Until now, four genotypes of CHIKV have been reported (Weaver, 2014), The Est Central South African (ECSA) genotype, West

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African genotype, Asian genotype and Indian Ocean Linage (IOL) genotype.

CHIKV was first reported as a human pathogen in 1952 in Africa, when the virus was isolated by Ross from a serum of human during an epidemic in Tanzania (Lumbsden, 1952; Ross, 1956). In 1958, numerous cases of chikungunya fever have been also identified in several countries in Asia. The significant outbreaks occurred between the 1960s and 1973 in Bankok and India (Nimmannitya et al., 1964; Shah et al., 1964; Padbidri et al., 1979; Jupp et al., 1988). Interestingly, the re-emergence of the virus has been reported between the 1960s and 1990s in several African countries such as Uganda, Zimbabwe, Senegal, Cameroon and Guinea (Williams et al., 1965; Halstead, 1969; Padbidri, 1979; Jupp, 1988; Lanciotti, 1998). In 2005, several cases of chikungunya fever were reported in La Reunion island, interestingly, the number of people infected have increased in 2006, more than 266,000 cases were documented (Chretien et al., 2007; Cire La Re´union-Mayotte, 2006). Numerous scientists suggested that the virus was introduced into La Reunion Island because of the movement of people from the islands of the Indian Ocean to this island. In addition, other researchers suggested that CHIKV was transmitted by *Aedes albopictus* and not via *Aedes aegypti* due to the limited numbers of *A. aegypti* on La Reunion Island (Reiter et al., 2006).

In 2007, chikungunya virus was detected in Italy for the first time, which means that the virus has been introduced into Europe causing a new outbreak, this finding suggested that CHIKV can move and affect new ecological niches in Europe and other countries such as Australia and countries in the Western Hemisphere (Rezza et al., 2007; Staples et al., 2009).

In January 2011, CHIKV was detected for the first time in the Eastern Mediterranean Region of the World Health Organization when the Ministry of public health and population of Yemen reported several numbers of Dengue-like illnesses in AL-Hudaydah governorate in Yemen. Since, numerous researches have been carried out to investigate the origin of this outbreak. Unfortunately, the epicenter of this outbreak is still unknown; however, this outbreak was completely curtailed (Malik et al., 2014).

In 2012, another study was performed in Yemen to investigate the co-circulation of Dengue and CHIKV. In this study, the sera of 400 patients with dengue-like illness symptoms were studied using immunological and molecular technique. Among the 400 patients, 116 (29%) were positive for dengue virus, whereas 49 (12%) were positive for CHIKV (Rezza et al., 2014) the results obtained demonstrated that mosquito-borne infections in Yemen represent a serious public health threat.

In 2015, the complete genome of CHIKV was sequenced by Fahmy et al. (2015); this virus was isolated from an *A. aegypti* mosquito during the outbreak in Yemen in 2011. In this work, genome analysis showed that CHIKV isolate represent significant similarity with the Indian oceans strains (Fahmy et al., 2015).

Recently, another outbreak occurred in 2012 in the Philippines (Tan et al., 2015). In this study, scientists studied the phylogenetic relationship of CHIKV isolate obtained during the Philippines outbreak with numerous Chikungunya viruses sequences isolated from different regions in China, Micronesia and Caribbean. Interestingly, the results obtained suggested independent emergence of CHIKV in the Philippines, which then extend into China, Micronesia and the Caribbean region. Few years later, CHIKV has re-emerged in 2014 causing new outbreak in Puerto Rico and Brazil (Chiu et al., 2015; Nunes et al., 2015).

Objective

The present study aimed to construct an updated phylogenetic tree of currently published data and perform a phylogeographic analysis of CHIKV obtained during different outbreak in the last five years after the re-emergence chikungunya virus to get further insight into the epidemiology and transmission of CHIKV.

MATERIALS AND METHODS

Collection of E1 gene sequences

Twenty two sequences from the E1 envelope glycoprotein gene were collected and retrieved from the National Center for Biotechnology Information (NCBI) available (https://www.ncbi.nlm.nih.gov/). These twenty two sequences were published recently after the re-emerging of CHIKV in Europe, Asia, Africa, as well as the Eastern Mediterranean Region of the World Health Organization. The retrieved sequences were from Yemen outbreak- KJ742803- KJ742804- KJ742805- KJ742806- KJ742807- KJ742808- KJ742809 (Rezza et al., 2014), Italy outbreak- KM267638 (Rossini et al., 2016), Philippines outbreak- KM014692- KM014693 KM014694- KM014695- KM014696 (Yoon et al., 2015), Philippines outbreak- KP276677 (Velasco et al., 2015), India outbreak- KX358423- KX358422- KX358421- KX358419- KX358417- KX358410- KX358408 deposit in GenBank by Khan and Ray (unpublished) and from Tanzania outbreak- AF192905 deposit in GenBank by Logue and Atkins (unpublished).

Phylogenetic tree and sequences analysis

The 22 sequences were aligned using ClustalW software program (http://www.ebi.ac.uk/clustalw2/). Phylogenetic trees was constructed by using MEGA 5 software version 6 (Tamura et al., 2011), to determine the phylogenetic relationships.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.14740026 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. All

Table 1. Localization and sample size of chikungunya virus.

Organism	State	Sample size
Chikungunya Virus	Yemen	7
Chikungunya Virus	Italy	1
Chikungunya Virus	Philippines	6
Chikungunya Virus	India	7
Chikungunya Virus	Tanzania	1

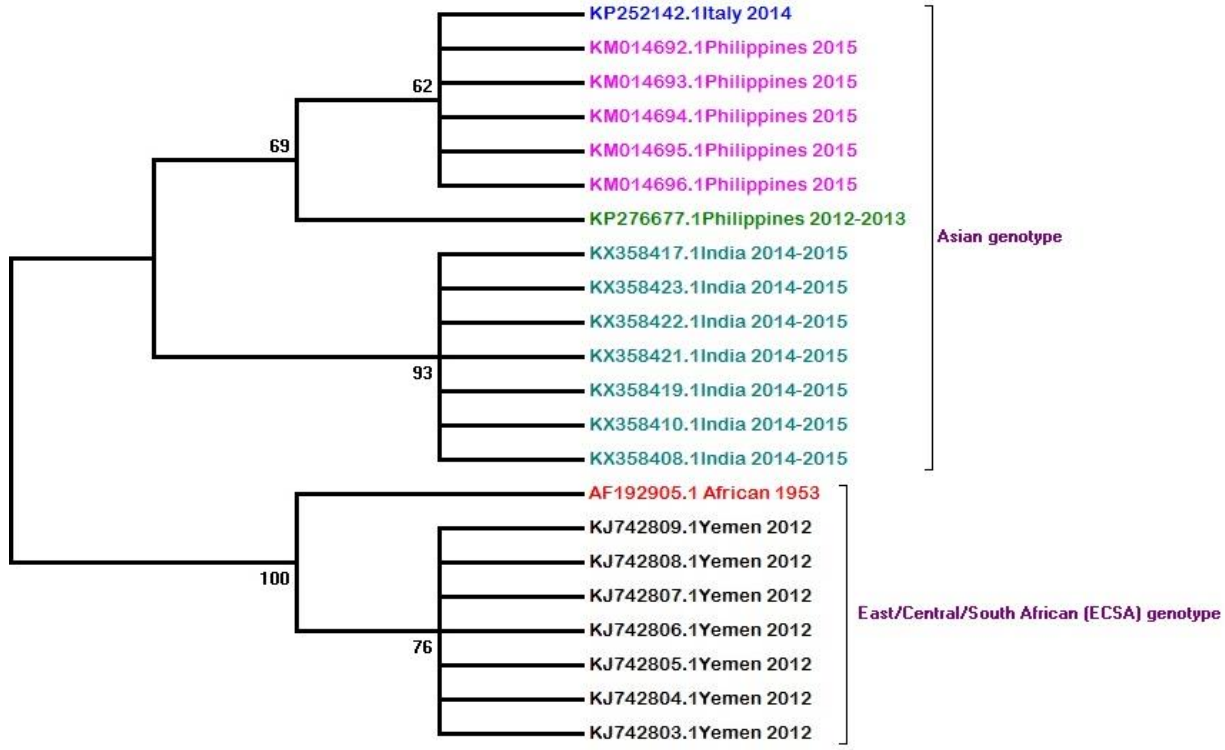

Figure 1. Evolutionary relationships among twenty two sequences of chikungunya virus obtained during different outbreak that occurred in the last five years based on the neighbor-joining (NJ) algorithm. Bootstrap values are marked on the branches

positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

RESULTS

In this study, phylogenetic analysis were performed and an updated phylogenetic tree was constructed comparing twenty two sequences of CHIKV strains from Yemen, Italy, Philippines, India and Africa. All sequences were published recently and were obtained during different outbreak in the last five years after the re-emerging of CHIKV. Information regarding sample size and localities are listed in the Table 1.

The phylogenetic tree was constructed by using neighborjoining (NJ) algorithm (Figure 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

The results obtained from this model indicate that among 22 CHIKV obtained from different states, CHIKV strains isolated during the outbreak in Yemen in 2011 show close relationship and form one group. Interestingly, this group share high similarity with CHIKV isolated in Tanzania in 1953.

In addition, among the six sequences obtained during Philippines outbreak, five sequences showed a close relationship with CHIKV isolated in Italy in 2014 and formed one group. On the contrary, CHIKV isolated in Philippines in 2012 was disclosed to this group.Sequence

analysis of CHIKV strains isolated during the outbreak in India in 2014 and 2015 demonstrated that the seven sequences share high similarity and form one group. In addition, this group show close relationship with the group of CHIKV isolated recently in Philippines.

DISCUSSION

CHIKV is known to cause an acute onset of high fever, severe arthralgia and rash, and is usually accompanied by headache and severe joint pain. This virus was first reported as a human pathogen in 1952 in Africa (Lumbsden, 1952; Ross, 1956). Interestingly, Chikungunya virus has re-emerged recently in new areas, and numerous outbreaks occurred in different states in Europe, Asia, America and Africa. These mosquito-borne infections represent a serious public health threat. CHIKV is principally transmitted to humans via the bite of an infected anthropophilic vector *A. aegypti* and *A. albopictus* (Centers for Disease Control and Prevention, 2011).

Unfortunately, until now, there is no vaccine for CHIKV. The control of the disease mainly remains dependent on the control of the vector. Furthermore, many researchers have demonstrated that CHIKV transmission is mediated by vectors that can colonize new geographical area due to its capacity to acclimatize to different climates. This can explain why the rate of infection has recently increased dramatically especially in tropical countries. In addition, the return of peoples from affected areas is also one of several raisons that explain the detection of CHIKV outside tropical countries (Presti et al., 2014).

In the present work, an updated phylogenetic tree was provided, the results demonstrated that CHIKV strains isolated recently in the Eastern Mediterranean Region share high similarity with chikungunya virus isolated in Tanzania in 1953.

Unfortunately, the epicenter of many outbreaks is still unknown, however, some of these outbreaks were completely contained. To conclude, phylogenetic analyses of virus sequences are important tools to get more insight into the epidemiology and transmission of CHIKV. Moreover, several phylogeographic studies are needed to know and determine the epicenter of many outbreaks that occurred recently.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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