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# Symbiotic microorganisms of *Nasutitermes octopilis* (Blattodea: Termitoidae: Termitidae: Nasutitermitinae)

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## Declaration

I declare that this thesis entitled Symbiotic microorganisms of *Nasutitermes octopilis* (Blattodea: Termitoidae: Termitidae: Nasutitermitinae) is my own work and all the sources have been quoted and acknowledged by means of complete references.

In Prague *27.4. 2017* **…….……**

Bc. Patrik Soukup

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## Abstract

Termites are the most important decomposers of dead plant matter at the global scale. The dead plant material is rich in lignocellulose, which is decomposed by termites and their allied symbiotic microorganisms. While composition of endosymbiotic communities living in termite gut was already studied in series of termite species, the only known example of ectosymbiotic association is fungus-growing termites (Termitidae: Macrotermitinae) cultivating in their nests Termitomyces spp. (Basidiomycetes: Agaricales) fungi. The interactions and relationships among the host and the members of symbiotic assemblages are still unclear. In this study, I identify the structure bacterial and fungal communities in Nasutitermes octopilis (Blattodea: Termioidae: Termitidae: Nasutitermitinae) and its food source, using amplicon libraries of ITS2 and 16S rRNA genes. All amplicons were sequenced using Illumina MiSeq platform, and yield in total 1516197 bacterial sequences and 686016 fungal sequences. The sequencing yielded in total 116 bacterial and 45 fungal OTUs (Operational Taxonomic Unit) using public databases. The majority of bacterial OTUs belong to phyla Spirochaetes almost by 70 %, Fibrobacteres, candidate phylum Termite group 3 and Bacteroidetes. The bacterial taxa do not provide a clear pattern of distribution between termite and its food, and seems to be mostly species dependant without changes on structure. The fungal composition seems to be affected mostly by a time and environmental influence. But we found a species of fungus with possible association to termites. Fungus from Zygomycota phylum is relatively wide spread among colonies and it is clearly connected to the termites. The nature of these associations will be further examined during my Ph.D. studies.

**Key words:** termites, Isoptera, Termitoidae, Nasutitermes, symbiotic microorganisms, fungi, bacteria, molecular biology, PCR, NGS, Illumina

## Content

1. Introduction ..... 1
1.1 Taxonomy and diversity of termites ..... 1
1.1.1. Castes, ontogenesis and behavioural ecology of termites ..... 6
1.1.2. Ecology and life-types ..... 10
1.1.3. Termite feeding behaviour ..... 12
1.1.4. Termite digestive tract structure ..... 14
1.1.5 Cellulotic system in termites ..... 16
1.2 Symbotic organisms of termites ..... 17
1.2.1 Archae ..... 19
1.2.2. Bacteria ..... 19
1.2.3. Flagellates ..... 22
1.2.4. Fungi ..... 23
2. Aims of the Thesis ..... 24
3. Material and Methods ..... 25
3.1. Sampling ..... 25
3.2. Amplification by polymerase chain reaction (PCR) ..... 27
3.3. Electrophoresis ..... 28
3.4. Purification ..... 28
3.5. Measuring the DNA concentration ..... 28
3.6. Sequence analysis and OTU identification ..... 28
3.7. Data analysis ..... 29
4. Results and discussion ..... 30
4.1. Results and discussion for bacterial community ..... 30
4.1.1 Non-metric multidimensional scaling (NMDS) ..... 33
4.2. Results and discussion for fungal community ..... 36
4.2.1 NMDS analysis for ITS ..... 39
5. Conclusion ..... 42
6. References ..... 43

## List of figures

*Figure 1: Appearance of* N. octopilis*. Soldier at right-down, small worker right-up, and large workers at left of the photo (used with permission from Jan Šobotník).* ..... 5

Figure 2: Examples of ontogenesis between "higher" (left) and "lower" (right) termite. v: egg; L: larvae; mL: small larvae; mD: small worker; BV: white soldier; V: soldier; Ny: nymph; I: imago; Neo: neotenic rep. (Modified from Hanus & Šobotník 2004). ..... 6

Figure 3: A royal couple of Macrotermes carbonarius. The queen and the king (middle up); note the dimorphic workers and soldiers (used with permission from Jan Šobotník). ..... 8

Figure 4: Examples of soldiers. On the left: Hodotermopsis sjostedti Holmgren 1911; on the right: Termes sp. (used with permission from Jan Šobotník) ..... 10

Figure 5: Mound ("Cathedral nest") of Australian termite Nasutitermes triodae in comparison with full grown men (used with permission from Jan Šobotník). ..... 11

Figure 6: Digestive and enzymatic system in N. takasagoensis. Adopted from Ni and Tokuda(2013).Wood fragments are mixed with a β-Glucosidase from salivary gland are broken In the foregut to 150 µm -200 µm particles in diameter. The midgut secretes EGs and BGs into the lumen. The partially digested wood particles are moved to the hindgut trough the mixed segment. PM, peritrophic membrane. ..... 17

Figure 7**:** Fungal comb of Odontotermes sp. with a worker feeding on Termitomyces spp fungi (used with permission from Jan Šobotník). ..... 21

Figure 8 : Termitomyces sp. fruiting body growing out from Pseudacantotermes militaris fungal garden (Photography provided by courtesy of Jan Šobotník and Aleš Buček). ..... 24

Figure 9: The samples during the process of preparation, before the RNAlater is introduced into the 2 mL vials with termite food source (left) and control (right). David Sillam-Dussès is at the back inspecting the log from which the samples originated (used with permission from Jan Šobotník). ..... 26

Figure 10: Relative bacterial phyla abundances shown for an each type of samples. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. 32

Figure 11: Relative abundances for a bacterial distribution within a termite samples. .....33

Figure 12: **Non-metric multidimensional scaling** of the bacterial composition based on the termite samples. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies ..... 33

Figure 13: **Non-metric multidimensional scaling** of the bacterial composition based on the termite samples shown on the tree trunks. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. ..... 35

Figure 14: **PCoA** analysis of bacterial composition based on termite samples. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. ..... 35

Figure 15: Fungal relative abundancies within a sampled trunks for each type of sample. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. 37

Figure 16: Relative fungal phyla abundances shown for an each type of samples. ..... 38

Figure 17: **Non-metric multidimensional scaling** with normalised fungal abundances based on type of the sample (normalized data). Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. ..... 39

Figure 18: **Non-metric multidimensional scaling** with normalised fungal abundances based on the type samples for individual trunks (T). Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. ..... 40

Figure 19**: PCoA** analysis with absolute fungal abundances based on the type samples for individual trunks (T). Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies. ..... 41

## List of tables

Table 1**: taxonomic classification of termites** ..... 1

# 1. Introduction

## 1.1 Taxonomy and diversity of termites

*Table 1**: taxonomic classification of termites***

| | |
|---|---|
| **Kingdom** | **Animalia** |
| **Phylum** | **Arthropoda** |
| **Class** | **Insecta** |
| **Order** | **Dictyoptera** |
| **Infraorder** | **Blattodea** |
| **Epifamily** | **Termitoidae** |

Termites, together with a cockroaches and mantises form the order Dictyoptera (Inward et al., 2007). Modern Dictyoptera evolved in a relatively short time in the Mesozoic period (Nalepa and Bandi, 2000) and not earlier than in the Upper Jurassic (Thorne et al, 2000).

Termites form traditional infraorder Isoptera, but should be treated as epifamily Termitoidae (Inward et al., 2007), as their sister group are wood roaches Cryptocercidae (with a single genus *Cryptocercus*; Inward et al., 2007, Krishna et al., 2013). The last common ancestor of termites and *Cryptocercus* occurred in Upper Jurassic (Bourguignon et al., 2015). *Cryptocercus* share several apomorphies with termites, such as the diet based on the wood, flagellates which aid cellulose digestion, food and symbiont exchange by trophallaxis and biparental care (Inward et al., 2007).

A single species of family Mastotermitidae, *Mastotermes darwiniensis* Froggatt, 1897 lives Australia, although several other Mastotermitidae genera are known from the world-wide fossil record (Krishna et al., 2013). *M. darwiniensis* can also be found on a New Guinea where was probably introduced by man (Lewis, 2009; Evans et al., 2013).

*Mastotermes* is the most basal lineage of termites, and shares several plesiomorphies found only within cockroaches, such as like hind-wing anal lobe and wings venation, intracellular *Blattabacterium* symbionts, presence of ovipositor, and eggs laid in ootheca (Lewis, 2009; Eggleton, 2011, Lo and Eggleton, 2011). Ootheca may contain up to 24 eggs in two lines (Lewis, 2009). *Mastotermes* lives in very numerous colonies with the population reaching up to hundreds of thousands of individuals (Krishna et al., 2013)

Termites form small but a very abundant group of insects, with about 3000 species grouped into 9 families, and few hundreds of fossils species (Krishna et al. 2013). The early fossils often represent groups basal to *Mastotermes* or to the living families, especially Hodotermitidae (Engel et al., 2009) and Archotermopsidae (Thorne et al., 2000). During the last six decades, there was more than 1500 newly described species of termites (Krishna et al., 2013), and we can still expect that between 500 and 1000 additional species will be described (Eggleton, 2011).

Termites are commonly split into two informal groups, the “lower” termites comprising 8 following families: Mastotermitidae, Archotermopsidae, Stolotermitidae, Hodotermitidae, Kalotermitidae, Stylotermitidae, Serritermitidae, Rhinotermitidae and the “higher” termites, the Termitidae (Engel et al., 2009). The “higher” termites form 80% of termite generic diversity (Krishna et al., 2013)

**Archotermopsidae** (damp-wood termites) comprise of three genera, *Archotermopsis*, *Hodotermopsis* and *Zootermopsis* with altogether six species (Krishna et al. 2013). Members of this family can be found in the southern part of Himalayas (*Archotermopsis*; Roonwal et al, 1985), mountains of south China, Vietnam and Thailand, on Taiwan and surrounding islands (*Hodotermopsis*; Belyaeva 2004a), and in the western part of the North America (*Zootermopsis*; Krishna et al., 2013). All species are large and live in small colonies.

**Stolotermitidae** include two genera with a Gondwanian distribution, namely south Australia, Tasmania, New Zeeland, south Africa and south of South America

(Eggleton, 2000). The fossils of Stolotermitidae are known from the Early Miocene (Kaulfuss et al., 2010).

**Hodotermitidae** (harvesting termites) comprise of three genera and 21 species (Krishna et al., 2013), which are distributed in the arid and semi-arid regions of Africa, Middle East and India. All castes of this family are large and have developed eyes (Lewis, 2009). They are grass-feeders with preference to dry grass, but feeding also on shrub and tree material or living grass what makes them occasional pests of agriculture (Rouland-Lefèvre, 2011; Symes and Woodborne, 2011).

**Kalotermitidae** (dry-wood termites) includes 21 genera and more than 450 species (Krishna et al., 2013) with a wide geographic distribution, from South Europe to tropical forests on all continents (Lewis, 2009; Krishna et al., 2013). They are in general bigger in body size (Lewis, 2009), live in small colonies, and comprise important pest species (*Cryptotermes*, *Incisitermes*).

**Stylotermitidae** with a single genus and 45 species in a South-East Asia belong to the least known taxa (Krishna et al., 2013). Very few facts known about this genus is mostly due to their specialization on living trees. The unpublished molecular study suggest Stylotermitidae as the basal group of Neoisoptera, containing also all other families mentioned below (Šobotník, pers. comm.).

**Serritermitidae** comprise only two genera (*Serritermes* and *Glossotermes*), with altogether three species (Krishna et al., 2013). They are small in body size, live in small colonies and occur only in South America. *Serritermes* lives as *Cornitermes* inquiline, while *Glossotermes* feeds on rotten wood (Šobotník, pers. comm.).

**Rhinotermitidae** (subterranean termites) include 12 genera and 315 species of cosmotropical distribution (Krishna et al., 2013). They are small (e.g. *Termitogeton*) to mid-sized termites, and some species live in small colonies counting hundreds of members (e.g. *Termitogeton*, *Prorhinotermes*), while others in numerous colonies made of millions of members (some *Reticulitermes* and *Coptotermes*). *Reticulitermes* and *Coptotermes* belong to the pests with the most destructive potential. Rhinotermitidae

are polyphyletic assemblage, in which both Serritermitidae and Termitidae are nested (Bourguignon et al., 2015).

**Termitidae** contains more than 2000 termite species (approx. 80 % of known termite species) grouped into 238 genera (Krishna et al., 2013). Their diet is the most variable of all termite families, and includes soil, wood in different stage of decomposition or fungal mycelium and nodules of Termitomyces in Macrotermitinae (Batra and Batra, 1979; Rouland-Lefevre, 2000; Aanen et al., 2002). The family is split into 8 subfamilies, of which Nasutitermitinae, as a crown group, belongs to the most recent and the most successful ones (Krishna et al., 2013).

***Nasutitermes*** is the most speciose genus with cosmotropical distribution, comprising some 250 species (Krishna et al., 2013). At the same time, it is clearly polyphyletic (see Bourguignon et al., 2017), and will surely be split into series of monophyletic genera. Because of the large abundance, world-wide distribution and pest status of several species, it belongs to the most studied termite taxa (Rouland-Lefèvre 2011; Krishna et al. 2013). It is in particular *Nasutitermes corniger* Motschulsky, 1855, which is considered to be the most important pest of this genus, mainly in South America (Evans et al., 2013; Boulogne et al., 2017). It is native to the southern Mexico to the Argentina, but already invaded Florida, New Guinea and Bahamas (Evans et al., 2013).

*Nasutitermes* species always build centralised nests, which are usually arboreal, but epigeal and hypogeal nests are known in quite some species, too. The termites belong to smaller ones, and they live in abundant colonies that may contain up to hundreds of thousands of individuals or even few millions in some Neotropical species. For *Nasutitermes* is typical sexual dimorphism in worker's caste, where the females are larger than the males (Lima et al., 2013). Soldiers are usually males and may represent up 20 % of the colony population. Their head is typical for its bottle-like appearance, with the cone-shape nasus containing the defensive frontal gland that opens at the nasus tip, and reduced mandibles (Šobotník et al., 2010). The defensive secretion of soldiers consists of non-polar monoterpene hydrocarbons and polyoxygenated diterpenes, with high inter-specific and intra-specific variability in the composition of the frontal gland

secretion (Prestwich, 1979, 1984; Šobotník et al., 2010). *Nasutitermes* species consume a wide spectrum of plant materials, such sound and decayed wood, grass, or leaf-litter (Scheffrahn et al. 2005). The composition of symbiotic community was studied in *N. corniger*, in which the gut community comprises mostly Spirochetes (genus *Treponema*) and Fibrobacteres, i.e. termite group (TG) 3 (Warnecke et al., 2007; He et al., 2013).

***Nasutitermes octopilis*** Banks, 1918 is a common Neotropical termite, which feeds on sound or slightly decayed wood items. Unlike most of other Neotropical *Nasutitermes* species, it builds large epigeal nests made of hard clayish material, and a mature nest may comprise over a million of members. Although the termite is fairly common in virtually everywhere in French Guyana, seeing the nest is relatively rare event (Šobotník, pers. comm.).

*Figure 1: Appearance of* N. octopilis*. Soldier at right-down, small worker right-up, and large workers at left of the photo (used with permission from Jan Šobotník).*

### 1.1.1. Castes, ontogenesis and behavioural ecology of termites

Termite ontogenesis is based in hemimetabolous development, with series of similar instars, and step-by-step enlargement of body and wings (Rosin and Korb, 2011). There are two basic developmental patterns, the linear and the bifurcated model. The first is straight linear pathway where the development goes from the larvae through the nymphs and ending on the alate imago. The soldiers and neotenic reproductives develop as a deviation of this linear pathway (Noirot and Pasteels, 1987).

All undifferentiated individuals, i.e. larvae, pseudergates (false workers) and nymphs have potential to mature to an alate imago stage, and attempt to establish own colony if the food source is exhausted or under pressure from competitors (Rosin, 2015). This strategy is characteristic to Kalotermitidae and some other taxa among "lower" termites (Abe 1987, Shellman-Reeve 1997).

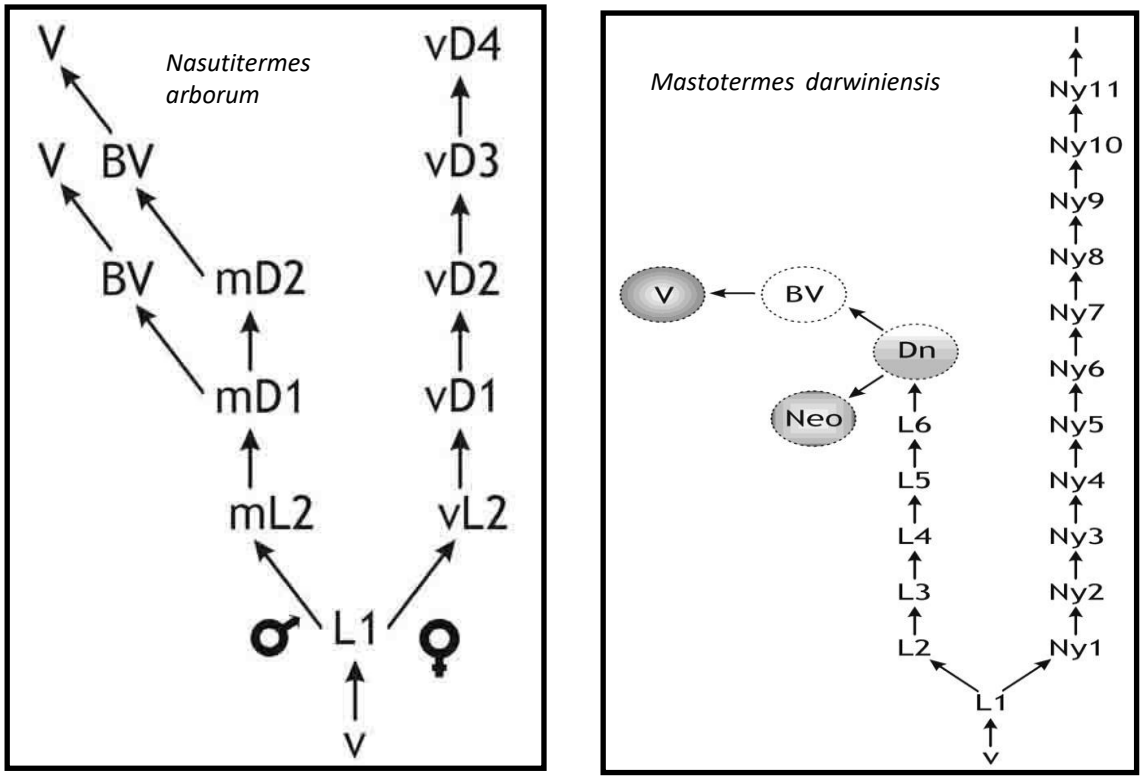


Figure 2: Examples of ontogenesis between "higher" (left) *Nasutitermes arborum* Smeathman, 1781 and "lower" *Mastotermes darwiniensis* (right) termite. v: egg; L: larvae; mL: small larvae; mD: small worker; BV: white soldier; V: soldier; Ny: nymph; I: imago; Neo: neotenic rep. (Modified from Hanus & Šobotník 2004).

Bifurcated model is characterised by an irreversible “decision point”, at which the larva either starts to develop into winged reproductive through a series of progressive moults, or belongs to the apterous line of true workers and soldiers (Noirot, 1969). The soldier caste originates mostly from workers, or less commonly from larvae or nymphs. Neotenic reproductives are common in “lower” termites, but very rare in Termitidae (Roisin, 2001; Roisin and Korb, 2011). This developmental scheme occurs in *Mastotermes*, Hodotermitidae, most of Rhinotermitidae, and all Termitidae.

Termite colony is always headed by a pair of reproductive, the king and the queen. Primary reproductives are the founding pair, while secondary reproductives may develop as replacement or supplementary ones, especially in the “lower” termites (Roisin, 2001; Roisin and Korb, 2011). Primary reproductives establish the new colony after a nuptial flight from their respective parental colonies (Henderson and Delaplane, 1993; Korb and Hartfelder, 2008). Soon after landing, the alates shed their wings and female start to attract males using a powerful sex pheromone (Laduguie et al., 1994; Bordereau and Pasteels, 2011).

When the couple is formed, female leads it to a specific place, where the nuptial chamber is constructed. The morphological changes are marked especially in the queen, and imply degeneration of flying and mandibular muscles, compound eyes, and also considerable growth of ovaries and fat body helping to keep high egg production (Korb and Hartfelder, 2008; Noirot, 1969). Termite queens (along with ant queens) belong to the most fertile animals as they can produce eggs by and incredible speed of up to 1 egg per second kept for few decades (Roonwal, 1960).

*Figure 3: A royal couple of Macrotermes carbonarius. The queen and the king (middle up); note the dimorphic workers and soldiers (used with permission from Jan Šobotník).*

The labour in termite colonies is executed by sterile castes (Krishna, 1969). Soldiers are specialised defenders, which are so specialised to fulfilling the tasks connected to colony protection that they cannot feed themselves, and are fully dependent on care from workers. Workers or pseudergates are responsible for maintaining the gallery system, harvesting for food and digesting it, and feeding the dependent castes such as larvae, soldiers and reproductives (Korb and Hartfelder, 2008). Although workers are fully sterile in all "higher" termites, they keep reproductive potential in all termites with linear development, but also in some with bifurcated (*Mastotermes*, some Rhinotermitidae). They can also participate at the colony defence, especially in soil-feeding termites in which the soldier proportion decreases up to the caste disappearance or during the intraspecific conflicts of chemically-defended species possessing specific auto-detoxification mechanisms (Šobotník et al., 2010, 2014; Bourguignon et al., 2016).

The true worker caste occurs in the most basal termite species, *Mastotermes darwiniensis*, also in families Termitidae, Hodotermitidae, and Rhinotermitidae (Noirot and Pasteels 1987, Korb and Hartfelder, 2008). The pseudergates are the major work force in Kalotermitidae, Archotermopsidae, Serritermitidae, and genera Prorhinotermes, *Psammotermes* and *Termitogeton* (all Rhinotermitidae). In some cases, true workers may coexist with pseudergates developing by wing regression, as reported e.g. in *Reticulitermes* or *Nasutitermes* (Roisin and Korb 2011).

The evolution of a true soldier caste is a rare event, and took place only in termites and eusocial aphids (McLeish and Chapman, 2007). Soldiers are monophyletic in all termites, as evidenced by their presence in all fossil and current basal species, and their development through two moultings with a short-term presoldier stage intermediate between worker and soldier, and last between 10 and 20 days (Noirot, 1985; Korb and Hartfelder, 2008).

The sole function of soldiers is to protect the colony from predators and competitors (Thorne, 1982; Eggleton, 2011; Roisin and Korb, 2011). They are the most specialized caste with manifold anatomical changes of their bodies, mostly comprising bigger and highly sclerotized heads with enlarged mandibles (Krishna et al., 2013) or stopper-like heads (e.g. Kalotermitidae: *Cryptotermes*, *Calcaritermes*, *Glyptotermes*, or many Termitidae: Apicotermitinae: *Apicotermes*-group; (Miller, 1969, Prestwich, 1984, Quennedey, 1984, Šobotník et al., 2010). All soldiers of Nasutitermitinae rely exclusively on chemical weapons in the form of the frontal gland secretion, which is ejected from the head gland opening onto the opponent (Thorne 1982; Prestwich, 1984; Šobotník et al., 2010). At the same time, the mandibles in nasute soldiers are reduced into small plates with no functional significance (Noirot, 1969).

The proportion of soldiers varies among the species, but is usually counted in units of per cents. The basal groups rely in general rather on static warfare in the form of fewer soldiers considerably bigger compared to workers, while advanced taxa usually possess bigger numbers of soldiers smaller than workers. Another clear trend is decreasing the soldier-to-worker ratio in soil-feeding termites, and this trend resulted in

repeated loss of soldier caste in at least three termite lineages (Šobotník et al., 2010; Eggleton, 2011).

Termites represent common prey of various predators, from non-specialised opportunists to highly specialised ones able to overcome sophisticated termite defences (Deligne et al., 1981; Prestwich, 1984; Šobotník et al., 2010). Every tropical continent has a set of specialised mammal termite – and ant-eaters. The most important predators of termites are anyway ants, and we can observe an arm-race between these tropical superpowers (Deligne et al., 1981; Šobotník et al., 2010; Eggleton, 2011).

*Figure 4: Examples of soldiers. On the left: Hodotermopsis sjostedti Holmgren 1911; on the right: Termes sp. (used with permission from Jan Šobotník).*

### 1.1.2. Ecology and life-types

All termite species are eusocial, and live in colonies with reproductives, the queen and the king, and workers, soldiers and nymphs, i.e. brachypterous individuals on the way to alate imago (Eggleton, 2011). There is no single species of termites living solitary, all of them are eusocial (Lo and Eggleton, 2011).

Ability to build a nest is a characteristic feature of all eusocial insects. In termites, we can find really diverse set of nest types and structures (Noirot and Darlington, 2000). The nests are different among species, but also, we can often find large intraspecific variability (Roisin and Korb, 2011). Termite nest provides precise climate control in terms

of humidity and temperature for the good of the whole colony, but the nests are also protecting otherwise vulnerable termites from predators (Noirot and Darlington, 2000; Eggleton, 2011).

*Figure 5: Mound ("Cathedral nest") of Australian termite Nasutitermes triodae in comparison with full grown men (used with permission from Jan Šobotník).*

Based on the relationship between nest and the food sources, there are four termite life-types (Abe, 1987; Shellman-Reeve, 1997). **One piece type** (single-site nesting) comprises species living in a single piece of wood, which is a nest and exclusive food source for the whole colony. The consequence of this strategy is a short life span and small numbers of individuals in the colony, and is typical for Kalotermitidae or some Archotermopsidae and Rhinotermitidae. **Intermediate type** (multiple-site nesting) denominates habit wood feeding termites that do not build particular nesting structures, but are able to colonize new food sources through the subterranean galleries. This system is more stable compared to one piece type, and occurs in *Mastotermes*, or many

Rhinotermitidae (Abe, 1987; Shellman-Reeve, 1997). **Separate type** (central-site nesters) is characterized by presence of a centralized nest connected to foraging area by underground and/or aboveground system of galleries. The food of separate type termites is wood, as well as any other plant material in any stage of decomposition up to organic remains scattered in inorganic matrix of deeper soil layers. This strategy is characteristic for Hodotermitidae, some Rhinotermitidae and Termitidae (Abe, 1987; Shellman-Reeve, 1997). **Inquilinism** represent a strategy of living in the nest of another species (separate type), and feeding either on the food provisions or on the nest material itself. *Serritermes serrifer* (Serritermitidae) is the sole inquiline from
"lower" termites, and others belongs mostly among Termitinae (Termitidae; ShellmanReeve 1997).

Termites are one of the most successful invertebrates of humid lowland tropical areas. In these ecosystems, they may reach up to 95 % of insect biomass living in the soil (Eggleton et al., 1996; Eggleton, 2000; Jouquet, 2011). The impact of termites on their environment is so strong that they are often labelled as ecosystem engineers (Lavelle et al., 1997; Jouquet et al., 2006). Termites are together with fungi the most important decomposers of dead plant material as they are largely responsible for organic matter turnover and release of the key plant nutrients (Jouquet et al., 2006; Eggleton, 2011; Brauman et al., 2000; Donovan et al., 2001).

### 1.1.3. Termite feeding behaviour

The diet of all termite species is based on plant materials, and particular species can roughly be classified as herbivores, humivores or fungivores (Lewis, 2009). The lignocellulose is of recalcitrant nature, and animals possess a limited ability to break down the matrix. Majority of decomposition steps are provided by fungi and bacteria (Brune and Ohkuma, 2011; Ohkuma and Brune, 2011). Termite digestion is aided by rich microbial symbiotic communities (Breznak and Brune, 1994; Eggleton, 2000; Lo and Eggleton, 2011). The ability to process cellulose is around 74 % to 99 %, with the hemicellulose it is 65 % to 87 % (Breznak and Brune, 1994; Ohkuma 2003). In the tropical region termites consume from 40 to 100 % of dead plant material (Detling 1988; Bignell

and Eggleton, 2000). In a savanna biotopes, the 20 % of carbon mineralization is provided by termites (Detling, 1988; Bignell and Eggleton, 2000).

Termites could be devided into four (**I-IV**) ecological feeding groups, based on correspondence between the ingested food on one hand, and mouthparts and gut structure on the other (Donovan et al., 2001). Feeding group **I** comprises termites feeding on sound wood or grass, such as all "lower" termites do. All other feeding groups contain "higher" termites only. Feeding group **II** termites eat dead wood, grass and leaflitter. Feeding group **III** comprises species feeding on decayed wood, humus etc., while feeding group **IV** is reserved for true soil feeders eating soil organic matter without any discernible structure. Although the classification system provided by Donovan et al. (2001) allows an easy determination of the feeding group for whichever termite species, the fundamental difference is between wood-feeders (groups I and II) on one side, and soil-feeders (groups III and IV) on the other, as evidenced by broadly overlapping signatures of stable C and N isotope ratios (Bourguignon et al., 2011).

Overall, it is not so common among the animals to feed on soil, however in the termites, more than 60 % of species from all genera are humivorous (Brauman et al., 2000). So, it is not surprising, that in the tropical areas, termites play their important role in changing the soil quality (Eggleton, 2011). As soil is specific matter to feed on, therefore soil feeding termites exhibits differences in a physiochemical conditions of complexly structured gastrointestinal tract (Brune and Ohkuma., 2011). In comparison of foraged soil, the digested soil is enriched in concentration of carbon, nitrogen and the fulvic acids on the other hand the content of humic acids is reduced. (Brauman et al., 2000). The water content is also increased (Donovan et al., 2001) and he pH value is higher due to a high alkalinity in the soil feeding termite guts, which can be pH more than 12(Brune, 1998).

#### 1.1.3.1 Trophallaxis

The food exchange behaviour between two individuals can be divided into stomodeal or proctodeal, according to the end of digestive tract from which the donor provides the food. Trophallaxis is fundamental for termites as it satisfies the need of

nutrients for dependent castes, or symbionts and moisture to the newly moulted individuals (McMahan, 1969). Proctodeal trophallaxis can be observed only in the “lower termites” (Noirot and Noirot-Timotheé, 1969). It is a transfer of the gut fluid, stored in the rectal pouch, to the mouth of the receiver termite from the colony. It provides transfer of gut symbionts and also nutrients for the termites. It’s a way to refresh symbionts for the termite after the moulting, where these symbionts are lost (Ohkuma and Brune, 2011).

### 1.1.4. Termite digestive tract structure

Termite gut is divided in to three segments, foregut, midgut and hindgut. While forgut is a place of food moistening and grinding, the midgut is the major source of innate digestive enzymes, and hindgut is formed by a large paunch where the symbiontaided fermentation runs; further segments of hindgut serve for the absorption of nutrients (Breznak and Brune, 1994). In comparison with most of the “higher” termites, the gut of the “lower” termites are usually shorter and not so diverse, as it resemble mostly a cockroach gut or the gut of fungus growing termites of subfamily Macrotermitinae (Eggleton, 2011).

The higher termite reveal dramatic increase in the gut compartmentalization, with the hind gut showing the highest degree of variation in its structure (Tokuda et al., 2001; Ni and Tokuda, 2013).

The foregut is basically just a narrow tube of ectodermal origin, consists of welldeveloped muscles and unistratified epithelium covered with a cuticle. It consists of pharynx located in the head, and thoracal portion divided into long oesophagus ending in crop, followed by gizzard where the food is grinded have more developed muscular tissue, and the epithelium is more pronounced, with a different lobes and folds. (Noirot and Noirot-Timotheé, 1969). In the foregut, the food is transported to further sections and mixed up with digestive enzyme originated in the salivary glands (Bignell, 2011).

The midgut is a tubular structure, mostly with the unchanged diameter and structure lengthwise. The layer of muscles consists a curcilar and longitudinal fibres. (Noirot and Noirot-Timotheé, 1969). The epithelium composed of columnar cells releasing considerable part of digestive enzymes by merocrine secretion and regenerative cells (Tokuda et al., 2001). Malpighian tubes, as the sole excretory organs are attached at the midgut-hindgut junction (Breznak, 1994; Ni and Tokuda 2013). Mixed segment is a unique gut-part found only in advanced Termitidae. The mixed segment involves complicated border structure between midgut and hingut, to which also Malpighian tubules are integrated. The basic structure is similar we can find in the midgut. Half of the gut wall is made by mesenteric epithelium, same as in the midgut, but the rest of the gut is covered by proctodeal epithelium (Tokuda et al., 2001).

The hindgut is the largest gut part, and can be divided in to five segments, P1 to P5. The P1 segment, the ileum, is a large chamber where most of the food digestion takes place in wood-feeding termites, while other chambers of similar size often develop in soil-feeding species tubular part covered with a thick cuticle. This segment is also covered with an ornament of spines (Rocha and Constantin, 2015). The P2 segment, the enteric valve, is an important valve equipped with sphincter muscle, covered with a cuticular spines, pads and ridges, and its function is still debated (Donovan, 2002; Bourguignon et al., 2013; Kanazi and Ohmura 2016). The P3 segment, paunch is the longest part of the hindgut, occurring as a simple tube in wood feeders, while it can be further split into several enlarged chambers in soil-feeding species. Can comprise around ¼ of the gut length (Breznak, 1994; Tokuda et al., 2001; Rocha and Constantini, 2015). In some cases hindgut with symbionts can present around 40 % of weight of the termite (Slaytor et al. 1997) P4 segment, the colon is simple tube leading the gut content to the P5 segment, the rectum, where the last available nutrient and water are actively absorbed (Sousa et al., 2017).

Termite hindguts are the places where the most important digestive processes take place. As such, it provides stable microhabitats and food source for rich symbiotic assemblages comprising protists (only in “lower” termites), bacteria and archaea (Brune and Friedrich, 2000). These symbionts may form up to 40 % of fresh weight of a termite

(Slaytor et al., 1997). The plant organic matter decomposition is a process of fermentation, for which anaerobic conditions are required (Brune, 1998; Brune and Friedrich, 2000). Microelectrode studies discovered that hindguts reveal central anaerobic parts with a microoxic surrounding areas, where live aerobic and anaerobic microorganisms, respectively (Breznak, 2000; Brune and Friedrich, 2000).

### 1.1.5 Cellulotic system in termites

Termites, together with their symbiotic microbiota are one of the most efficient decomposer of plant biomass in the tropical and subtropical regions (Breznak and Brune, 1994; Ohkuma, 2003; Tokuda et al., 2004). The lignocellulose composition contain cellulose (20–50 %), hemicellulose (15–35 %), and lignin (18–35 %). Cellulose is the predominant component and the most abundant polymer on earth (Tome et al., 1995).

The cellulolytic enzymes are produced either by the termites or by their symbionts. Major source of them are flagellates occurring only in the “lower” termites, while they are of bacterial origin in the “higher” termites (Brune and Ohkuma, 2011). The three functional types of cellulases participate at the polymer to be split into digestible sugar. While endo-β-1,4-glucanases and β-glucosidases are secreted by both termites and symbiotic microorganisms, exoglucanases (cellodextrinases or cellobiohydrolases) originate only from the symbionts (Tokuda et al 2004; Ni and Tokuda, 2013). Interestingly, termite cellulases are of two origins even in termite bodies, as they are produced by labial glands in the “lower” termites, while midgut epithelium in the “higher” (Slaytor, 2000; Tokuda, 2004; Tokuda et al., 2011). The symbiotic communities have more complex cellulolytic enzymes in comparison to those of host. The main enzymes produced by symbiotic community are cellobiohydrolases GHF5 EGs, GHF7 EGs, GHF7, and CBHs (Ni and Tokuda, 2013).

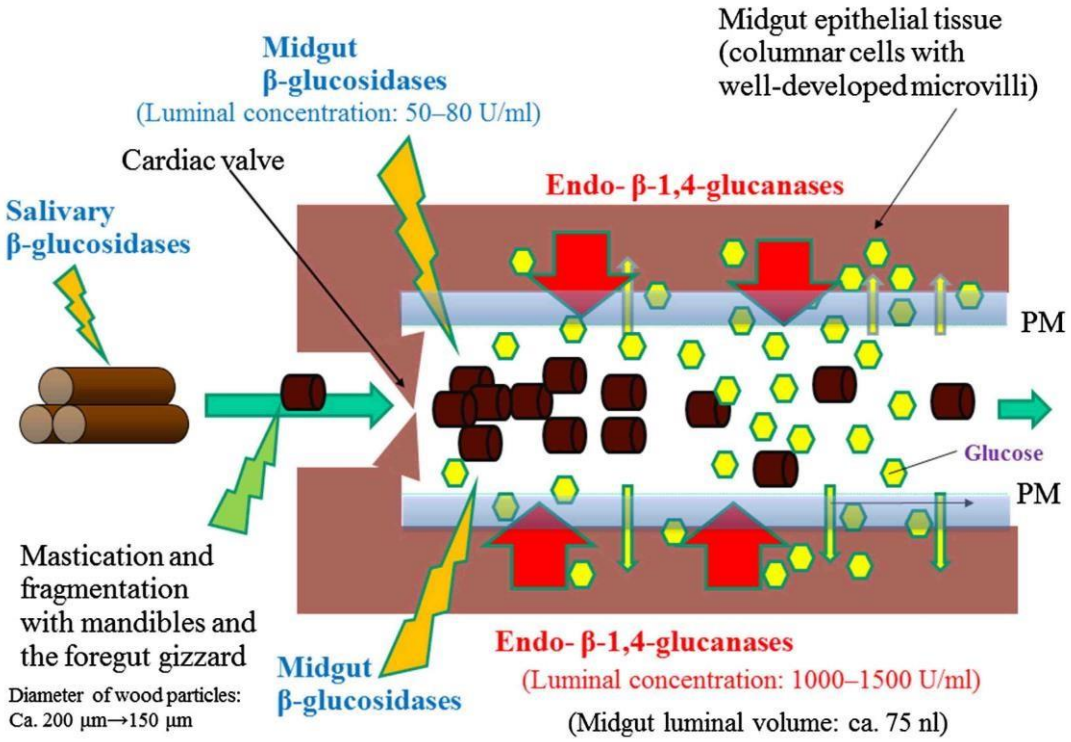


*Figure 6: Digestive and enzymatic system in N. takasagoensis. Adopted from Ni and Tokuda (2013). Wood fragments are mixed with a β-Glucosidase from salivary gland are broken In the foregut to 150 µm -200 µm particles in diameter. The midgut secretes EGs and BGs into the lumen. The partially digested wood particles are moved to the hindgut trough the mixed segment. PM, peritrophic membrane. Abbrevations: EGs endo-β-1,4-glucanases; BGs βGlucosidases*

Distribution of β-Glucosidases is also variable. In Nasutitermes spp. and several others is the β-Glucosidase secreted in the salivary glands, and the midgut (Tokuda et al., 1997; Slaytor, 2000). In other termites, it is almost strictly in the midgut (Slaytor, 2000). The Reduced activity of cellulases in the hindgut of *Nasutitermes takasagoensis* and *Nasutitermes walkeri* after an antibiotic treatment implies that the hindgut cellulases could have a bacterial origin (Tokuda and Watanabe, 2007)

## 1.2 Symbotic organisms of termites

The existence of diverse symbiotic population of microbiota in the termite gut is known for more than 80 years (Cleveland et al, 1935 Bignell, 2011). Most of this microorganism are difficult or even impossible to grow under artificial conditions and that's why our understanding remains limitedly understood (Ohkuma and Brune, 2011). However, the

Next Generation Sequencing (**NGS**) brings revolution to our knowledge in many fields of biology.

The most diverse and abundant microbiota occurs in the hindgut. The foregut and mid gut seems to be relatively poor in terms of microbial abundance and diversity (Hongoh, 2011). The functions of symbionts, summarized in Rouland-Lefèvre and Bignell (2001) are following: (**I**) dissimilatory carbohydrate metabolism of plant cell wall polymers, where the end products are shared between microorganisms and termite hosts. (**II**) Oxygen consumption, mainly at the gut periphery creates microoxic or anaerobic conditions in the central part of the gut, and provides thus suitable environment for fermentation and $N_2$ fixation process. (**III**) Dissimilatory and assimilatory N metabolism, using exuded uric acid as a source of nitrogen and assimilation of $N_2$ in an organic form. (**IV**) Hydrogenesis and hydrogen consumption by acetogenic or methanogenic symbionts. (**V**) $N_2$ fixation important mainly in wood feeding termites, which feed at nitrogen-poor substrate. (**VI**) Demethylation, decarboxylation and deacetylation of aromatic polymers. (**VII**) Humification of organic material and mineralization of carbon from proteinaceous component of humic acid, mainly in soil feeding termites.

We can characterize the symbiotic relationship between termites and diverse consortia of microorganisms as a nutritional mutualism. In this symbiosis plays the main role: Archaea, Bacteria, protozoa and fungi as a Eukarya (Bignell, 2000) and the dominant strains are unique to the host guts (Hongoh, 2011). Termites are highly dependent on their microbiomes, especially for the lignocellulose digestion (Ohkuma and Brune, 2011) and metabolism of nitrogen (Brune and Ohkuma, 2011).

The “lower” termites have in their gut bacteria and protozoa. Higher termites (Termitidae) gut contain no protozoa symbionts (Lewis, 2009; Lo and Eggleton 2011). The density of microorganism in the gut can reach up to $10^{11}$ cells/mL, where most of them lives in the hindgut (Ohkuma and Brune, 2011).

### 1.2.1 Archae

The three-main phylogenetic group, we can find in the termite guts are Methanobacteriales, Methanomicrobiales and Methanosarcinales, which form a unique Archaea cluster. Unlike other symbionts, the most methanogens are cultivable (Brune and Ohkuma, 2011) with a few exceptions (Leadbetter et al., 1998). Methanogens are generally attached to the hindgut wall or are associated with other microorganisms, like bacteria or flagellates as ecto or endosymbionts. This association to the flagellates may have some metabolic function or be a prevention to the washout (Brune and Ohkuma, 2011)

### 1.2.2. Bacteria

The origin of bacterial symbionts was a mystery for a long time. Hongoh et al., (2005) with his research shows, that some of the bacteria are specific symbionts which co-evolved with the termites regardless to a species, location, colony or individual. On the other hand, Dietrich et al (2014) results of comparison of termite and cockroach's bacterial symbionts, show that the origin is still unclear. And the lineages could be a diet specific acquired from environment or co-evolved from the ancestral cockroach. In most of the cases the bacterial lineages are unique to the termite gut, and in the 90 % the bacterial phylotypes are also unique and have no close relatives in the sequence database (Ohkuma and Brune, 2011).

The mixed segment of higher termite is occupied by the bacteria closely related to *Clostridium*, localized between peritrophic membrane and midgut wall (Tokuda et al., 2000). As obligate anaerobes, these bacteria plays role in a decreasing of oxygen in the mixed segment to preserve an anoxic environment in the hindgut (Brune et al., 1995).
Usually we can find three types of bacterial symbionts in the hindgut, epibiotic spirochetes, epibiotic and endobiotic rods (Dolan, 2001). The most abundant and typical bacterial symbionts are spirochaete (phylum Spirochaetes) (Brune, 2014). They can constitute more than 50 % of all bacteria in some species of wood feeding termites (Paster et al., 1996; Breznak, 2000; Hongoh et al., 2003; Brune, 2014). They are mainly

anaerobes inhabiting the hindgut of termites and a wood-roaches, freely moving in the fluid, attached to a protozoa (Paster and Breznak, 2015) or to the gut wall (Tokuda et al., 2001). Usualy almost imposible to cultivate, Graber et al. (2004), for the first time were able to cultivate the acetogenic spirochetes *Treponema azotonutricium* and *Treponema primitia.*

Another, a very abundant group are bacterias from Termite cluster I (Fibrobacteres) and Termite cluster III (TG3) (Mikaelian et al 2015). These Fibrobateres and bacteria from phylum TG3, together with a Spirochaetes are often found to colonize a wood fibres in the hindgut of wood feeding species. The Spirochaets are ubiquitous in the hindgut, however in the case of TG3 and Fibrobactres, those are exclusively associated to the wood particles. (Mikaelyan et al., 2014). Those fibre-associated bacterial symbionts are significantly participate on the cellulotic activity (He et al., 2013; Warnecke et al., 2007; Mikaelyan et al., 2014), but its ratio and composition could be changed with the artificial diet (Miyata et al., 2007). Therefore, in the humus feeding termites, which diet contain higher amount of lignocellulose, the fibre associated bacteria from Treponema cluster (Spirochaetes), Termite cluster I (Fibrobacteres) and Termite cluster III (TG3) are more abundant than in the soil feeders (Mikaelian et al 2015)

Ohkuma and Kudo (1996) discovered a new bacterial candidate phylum Termite group I (TG-I) from hindguts of *Reticulitermes speratus*. This phylum is very numerous and mostly are present as endosymbionts of flagellates in the hindgut (Hongoh et al., 2003; Herlemann et al., 2007), but have also no culturable representative (Ohkuma et al., 2007). Hongoh et al., (2008) suggest that these symbionts have crucial role by supplying their host protist and the termite with essential nitrogenous compounds deficient in lignocelluloses. Fixation of nitrogen by a symbiotic bacteria is another very important part of the nitrogen economy for the termite (Ohkuma, 2008). Novel TG1 group is probably only present in a lower termites and wood roaches Cryptocercus (Ohkuma et al., 2007). TG 2 is again a novel phylum, yet uncultivated and present in “higher” termites (Brune and Ohkuma, 2011). Novel termite group 3 phylum (TG3) are present in wood feeding higher termites and can consist of 10 % of all bacteria in the gut (Ohkuma and Brune, 2011).

The ecological relationship between bacteria and protist are still unclear. The endosymbiotic bacteria (Bacteroidales) can consist up over 80 % of the total bacterial rRNA and 70 % of the total bacterial cells on a protist *Pseudotrichonympha grassii* in a *Coptotermes formosanus* Shiraki, 1909 gut (Noda et al., 2005). These bacteria attached to protist, can help with motions of chemotaxis (Dolan, 2001) or they can be somehow affect the metabolism of their host (Radek and Nitsch, 2007)

In the Macrotermitinae are bacteria related to the fungal combs structure. Although Spirochaetes are common in termite's guts, on the fungal combs community they are rarely present. Commonly we can find species from Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Candidate division TM7 (Otani et al., 2014).

*Figure 7: Fungal comb of Odontotermes sp. with a worker feeding on Termitomyces spp fungi (used with permission from Jan Šobotník).*

***Blattabacterium***

*Blattabacterium* species are gram-negative symbiotic bacteria, distinctive for all cockroach families, except family *Nocticolidae* where was confirmed that this family is phylogenetically divergent from others (Lo et al., 2007a). But this symbiont can be also found in termite species *Mastotermes darwiniensis*, but no other termite species (Lo and Eggleton, 2011), probably because the role of *Blattabacterium* was replaced by the gut microbiota (Lo et al., 2007).

There was supposed that *Blattabacterium* consist only one species, *B. cuenoti,* but the recent DNA analyses of 6 species of *Cryptocercus* showed at least three new species: *B. relictus*, *B. clevelandi* and *Blattabacterium punctulatus* (Clark and Kambhampati, 2003).

### 1.2.3. Flagellates

The mutualism between flagellates and hosts occurred in the common ancestor of termites and *Cryptocercus*, between 150 to 170 million years ago (Mya), in Jurassic period (Inoue et al., 2000; Breznak and Brune, 1994; Dolan, 2001; Ohkuma, 2008; Lo and Eggleton, 2011; Ohkuma and Brune, 2011; Brune, 2014). These flagellates belong to phylum Parabasalia, orders Trichonymphida, Cristamonadida, Spirotrichonymphida, Trichomonadida, and from phylum Preaxostyla, order Oxymonadida (Adl et al., 2005; Ohkuma et al., 2007; Ohkuma and Brune, 2011). The flagellates were lost in ancestor of the “higher” termites, dated back to around 60 Mya in the Eocene (Bourguignon et al., 2015, 2017).

They are essential for all “lower” termites, as they intake wood particles by phagocytosis and degrade them (Honigberg, 1970; Breznak and Brune, 1994). The defaunation leads to a death in every individual, until they have a chance to reacquire the symbiotic community from nestmates (Cleveland, 1923). The set of flagellates could vary from 3 species in *C. formosanus up* to 19 species in *Hodotermopsis japonica* (Brune, 2014). Many of protest species reveal intimate relationship with their own specific symbiotic bacteria, which may live on or in their cells (ectosymbionts or endosymbionts of termite endosymbiont, respectively; Radek and Nitsch, 2007).

### 1.2.4. Fungi

Across the “lower” and “higher” termites, there often are certain interaction between fungi and termite (Nobre et al., 2011). The most intimate relation exists between *Termitomyces* spp. (Basidiomycetes: Agaricales) fungi and Mactotermitinae. This symbiosis between fungus *Termitomyces spp.* and Macrotermitinae is the eldest interaction (Nobre et al., 2011) and has a single origin on Africa continent (Aanen et al., 2002).

*Macrotermitinae* are highly abundant group of termites in Old World tropics. Workers are collecting dead plant matter which is partially digested by termites, the primary faeces are used to construct fungal gardens inoculated with asexual spores of fungi as it is passing through termite gut (Poulsen et al., 2014; Brune, 2014). This semi digested matter is further degraded by a *Termitomyces spp.*, and after the material exhaustion, the fungus comb is eaten by termites again, and deposited as terminal faeces to the protective outer nest wall (Grassé, 1982; Poulsen et al., 2014; Otani et al., 2016). Macrotermitinae alates do not bring the inoculum from parental colony, and the re-acquisition takes place by the first worker cohort to collect the sexual spores of the fungus from environment (horizontal transmission). However, in two unrelated genera of Macrotermitinae we can find a vertical transmission. Its provided by one of the reproductives which carries asexual spores from its colony, to set up a new fungus comb (Korb and Aanen, 2002) and to be a source of microbes for the first workers and soldiers. Many important genera of bacteria (*Alistipes, Bacteroides,* and *Desulfovibrio*) are common in Macrotermitinae workers and soldiers, but in a queen, they are missing. The more than 80 % bacteria in the queen gut belongs to the *Bacillus sp.* Diet of the queen is probably exclusively based on *Termitomyces* asexual spores (Poulsen et al., 2014).

Another interaction takes form of termite preference to certain degree of substrate decomposition, enhanced by presence of certain fungi which have positive effect on food discovery, consumption rate or content of available nutrients (RoulandLefevre, 2000; Cornelius et al., 2012). In this interaction termites feeding behaviour is affected by a species of fungus, also the level of decay of wooden is an

important factor (Cornelius et al., 2012). Except Macrotermitinae, in all other subfamilies of higher termites the fungal digestion is probably not important (Eggleton 2011, Brune, 2014).

*Figure 8: Termitomyces sp. fruiting body growing out from Pseudacantotermes militaris fungal garden (Photography provided by courtesy of Jan Šobotník and Aleš Buček).*

## 2. Aims of the Thesis

The aim of this work was to determine a spectrum of symbiotic microorganism in the termite *Nasutitermes octopilis* (Termitidae: Nasutitermitinae) and compare it with a composition of microbial community in their food source. These data we allowed us to test for the hypothesis that the termites are actively altering the microbial community within their food source. By comparing the communities occurring in termite food with the same wood devoid of any termite activity, we will decide about proportion of fungi

and bacteria spread by termites in their environment. These results will allow us to target specific taxa to be further studied within my Postgraduate studies.

# 3. Material and Methods

## 3.1. Sampling

The field work was performed by Jan Šobotník, Thomas Bourguignon and David Sillam-Dussès in November 2014, in Nouragues Nature reserve (French Guyana). All encountered wood items longer than 5 meters were inspected, and *Nasutitermes octopilis* termites were sampled for species identification (at least 10 soldiers and at least 10 workers put into 2 mL filled up with 80 % ethanol) and composition of symbiotic communities (10 workers put into 2 mL vial filled with RNAlater® fixative). Additional sampling comprised the food source (a piece of wood containing the terminal termite gallery) and the control to the food (the same wood 10 cm away from the closest termite gallery). All samples were taken with sterile tools, and the sterility was ensured by careful wiping of each tool on the cotton sheet treated with absolute alcohol. Three repetitions of the sample set, each of them placed at least 1 m away from the closest one, were prepared from each of studied logs (12 in total), in order to test for the fidelity in termite-microorganism association. The samples were taken during the day, and put into freezer in the evening, and they were transported to Czech Republic in the frozen state. In Prague, the samples were stored in -80 °C until further steps as described below.

*Figure 9: The samples during the process of preparation, before the RNAlater is introduced into the 2 mL vials with termite food source (left) and control (right). David Sillam-Dussès is at the back inspecting the log from which the samples originated (used with permission from Jan Šobotník).*

### 3.2. Homogenization and isolation DNA

To extract total DNA from termite samples, we used Macherey-Nagel NucleoSpin® Soil kit for DNA isolation according to the enclosed protocol, with few changes mentioned below. We used 5 randomly-chosen termite workers out of 10 in the sample, which were cleared from the residues of RNAlater® on clean paper tissue and homogenized together with a 500 µL of SL1 Lysis buffer and 100 µL of SX enhancer buffer, two sterilized steel beads 3 mm diameter using an oscillatory mill for 2 min, set on 30 swings per second. Sample lysis step was shortened to 2 min on vortexer. For precipitating contaminants was used 250 µL SL3 buffer.

For wood samples, Macherey-Nagel NucleoSpin® Soil kit was used for DNA isolation according to the manufacturer's protocol, with few changes mentioned below.

The samples of the wood were cleared from RNAlater® residues as described below. All wood in the sample was mechanically crushed (between each sample, the instruments were sterilized in autoclave to prevent possible contamination), placed in a sterile 2 mL tube with a 5 steel beads. To improve homogenization, all tubes were placed in liquid nitrogen for 1 min prior grinding in oscillatory mill. Grinding was set to 30 swings per second for 10 minutes and whole process was repeated until the wood matter turned into a powder texture 550 µL SL2 of extraction buffer was added to homogenized material and the grinding was repeated once more, without freezing in liquid nitrogen. Sample lysis vortexing was extended to 10 min. Precipitation of contaminants was done with 100 µL of SL3 buffer. Lysate was filtered with 650 µL of supernatant. Drying on silica membrane was for 3minutes in centrifuge and 1 min. of drying on air. Elution of DNA for medium concentration (adding 50 µL SE buffer) and centrifuge for 45 s.

## 3.2. Amplification by polymerase chain reaction (PCR)

Each PCR reaction contained: dNTP- 1µL [10mM equal dNTP mix], Buffer - 2,5 µL, BSA (Bovine serum albumine) - 1,5 µL $MgCl_2$ - 0,75 µL and dd$H_2O$ - 12,90 µL, 0,35 µL Polymerase DyNAzyme II DNA Polymerase (2 U/µL). 4 µL of the sample DNA isolate was added and 1 µL of each, reverse and forward primer, was added to the PCR. For each sample was used an original combination of tagged primers making the Illumina sequencing possible (see below).

Altogether, the mixture of one reaction was 25 µL in volume. Each PCR was repeated three times independently with the same primer combination, to neutralise possible random PCR bias.

PCR was performed at Eppendorf Mastercycler® nexus cycler. For amplification of the 16S gene, an initial denaturation at 94°C for 3 min, was followed by a thermocycling 94 °C for 45 secs, 50 °C for 1 min, and 72 °C for 45 sec (40 cycles) and a

final extension step at 72 °C for 10 min. Then, the samples were cooled to 8 °C continuously. The Reaction for fungal ITS 94 °C–5 min, 94 °C–45 sec, 50 °C–1 min, 72 °C – 45sec, 72 °C–10 min; 40 cycles and cooling to 8 °C continuously.

### 3.3. Electrophoresis

To assure that the PCR reaction was successful, the 5 µL of PCR products were mixed with a 2 µL Loading Dye Buffer and submitted to electrophoresis on 1 % agarose gel, containing ethidium bromide for DNA visualization. The horizontal electrophoresis run on 120 V for about 45 min. PCR product on gel was visualized by UV transillumination.

### 3.4. Purification

PCR product purification for sequencing purposes was done using MinElute PCR Purification Kit (250) (QIAGEN), according to enclosed protocol. For the better yields, the final step was done twice, using 2x10 µL of elution buffer.

### 3.5. Measuring the DNA concentration

For purposes of Next Generation Sequencing, purified PCR product concentrations were measured using Qubit® 3.0 Fluorometer with HS-dsDNA assay kit. For each measurement was used 2 µL of the PCR product.

### 3.6. Sequence analysis and OTU identification

Sequencing was done by Laboratory of Environmental Microbiology, Institute of Microbiology, Czech Academy of Sciences, using Illumina MiSeq platform. The obtained data were received filtered and with samples names added to each sequence. The data

The data obtained from Illumina MiSeq were submitted to the pipeline SEED2 (Větrovský and Baldrián, 2013). For the clustering in SEED2, I used Usearch-UPARSE (Edgar, 2013), and after clustering, the chimeric sequences were removed from the clusters as indicated by the software. For the purposes of cluster identification, the most abundant sequence from each cluster was used and OTU was defined with ≥ 97.0 %

sequence identity. Bacterial sequences were classified based on Ribosomal Database Project reference dataset (Wang et al., 2007, RDP Release 11, Update 5: September 30, 2016) and Fungal ITS sequences were classified based on UNITE (Kõljalg et al., 2013, 7.1 2016-11-20) reference database release supplemented by plant and animal sequences from GenBank to identify nonspecific sequences. OTU table was created after using SEED2 basic function.

### 3.7. Data analysis

For analysis of SEED2 outcomes and samples variables was used software R (KurtR version 3.3.3 (2017-03-06)) in environment of software R-Studio (ver. Version 1.0.136) and phyloseq package (McMurdie and Holmes, 2013)

# 4. Results and discussion

## 4.1. Results and discussion for bacterial community.

In order to determine a phylogenetic diversity among the microbial community in the termite guts, 16S rDNA clones were amplified from isolated DNA of termite *Nasutitermes octopilis*. From the total 1516197 sequences of 16S, 116 phylotypes (OTUs of 3 % genetic difference) were recognized. These phylotypes were classified into 11 bacterial phyla, 2 fungal phyla and 4 unclassified OTUs.

From the classified phyla, the Spirochaetes were the most dominant phylum among termite samples (see Figure 11). In total 68, 6 % of all bacterial sequences from termite samples were assign into 20 phylotypes identified as Spirochaetes. The majority falls into a family Spirochaetaceae but unfortunately no deeper classification was obtained from the available databases. Nevertheless, with high probability I assume that they are from genus *Treponema* cluster I (Ohkuma et al., 1999), which is the most abundant within the genus of Spirochaetes (Warnecke et al., 2007; Boulogne et al., 2016). Also, it is the most common phylum among wood feeding termites where it can reach up to 70 % of the 16S reads of the hindgut community in *Nasutitermes* spp. (Ohkuma et al., 1999; Hongoh et al., 2006; Köhler et al., 2012), what corresponds well with our results. Spirochaets were also recognized in the galleries of termites (12 %), perhaps caused by a random contamination of wood with the feaces of termites. A negligible amount of Spirochates (0,3%) were found in the control wood also, probably as a result of contamination during the sample preparation.

The second most abundant cluster of OTUs within termites was Fibrobacteres with 17 % of total sequence quantity, divided into 6 phylotypes. All of them belongs to the single family Fibrobacteraceae. The control wood was without a Fibrobacteres but the sequences obtained from galleries shows a presence of this cluster. One of the main feature of this phylum is the majority are anaerobic (Warnecke et al., 2007; Rahman et al., 2016) but there are some exceptions. Termite gut is not strictly anaerobic environment (Brune and Friedrich, 2000) and some of termite gut Fibrobacteres have respiratory chains adapted to the microaerobic conditions found in the termite gut

(Rahman et al., 2016). However, the occurrence of this phyla in a strictly aerobic environment was probably caused by a fresh contamination from termite feaces. From a termite samples, we obtained a several identified clones from TG3 phyla. Candidate phylum TG3 is often phylogenetically associated with the Fibrobacteres (Hongoh et al., 2005; Warnecke et al., 2007; He et al., 2013) and also morphologically resembles a small Fibrobacteres.

Another phylum connected to termites but less abundant is Bacteroidetes. This taxon accounts for 6 phylotypes in our termite samples, i.e. 2, 64 % of the overall bacterial diversity. OUTs belonging to Bacteroidetes were almost twice as abundant in the termite food compared to the control wood, what also means that some members of this group have capacities to penetrate into the woody material, a feature uncommon in mostly motionless bacteria. Although Bacteroidetes belong usually to the second most abundant group of bacteria, they accounted for between 2 % to 30 %, depending on species (Hongoh et al., 2006; Otani et al., 2014).

Proteobacteria found in termites were represent in 27 phylotypes but only in a few sequences (1 %) across all termite samples, and together with Actinobacteria, Cyanobacteria, Chlorobi and Chloroflexi, their abundance is insignificant. Phylum Firmicutes, mostly order Clostridiales, were also represented only by a few sequences (0,5 %), while their abundance was reported higher, usually around 10 % in other woodfeeding termites (Hongoh et al., 2006). Most of these low abundant phyla obtained from the termites, reached higher abundances in the control samples, and in the galleries as well. We can therefore mark all these OTUs as residents in the food source.

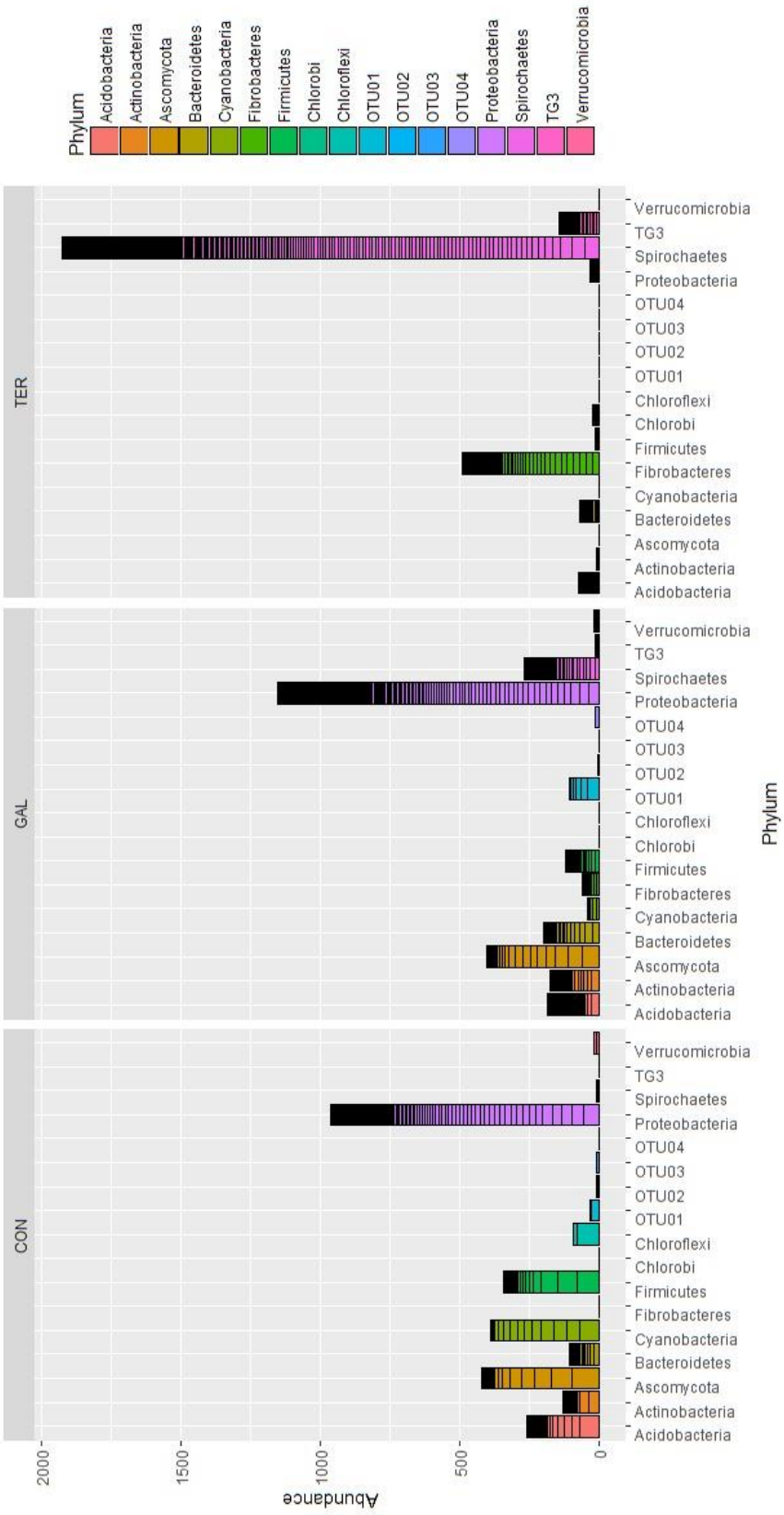


*Figure 10: Relative bacterial phyla abundances shown for each type of samples. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

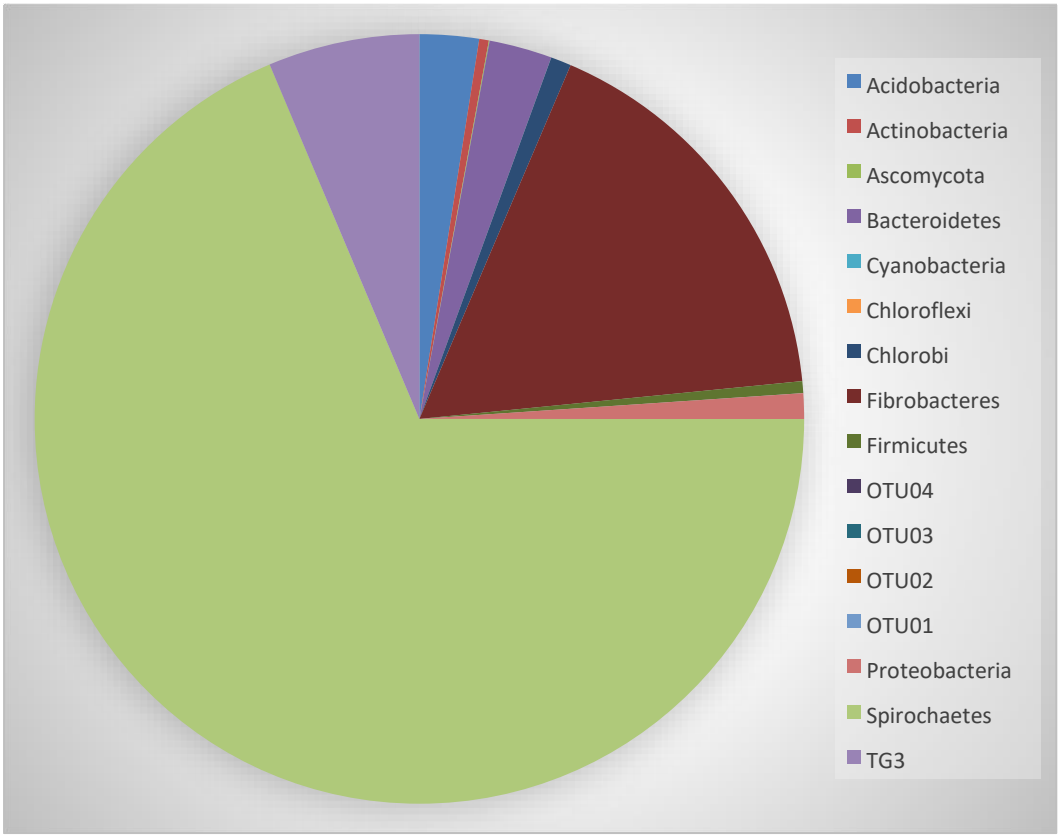


*Figure 11: Relative abundances for a bacterial distribution within a termite samples.*

### 4.1.1 Non-metric multidimensional scaling (NMDS)

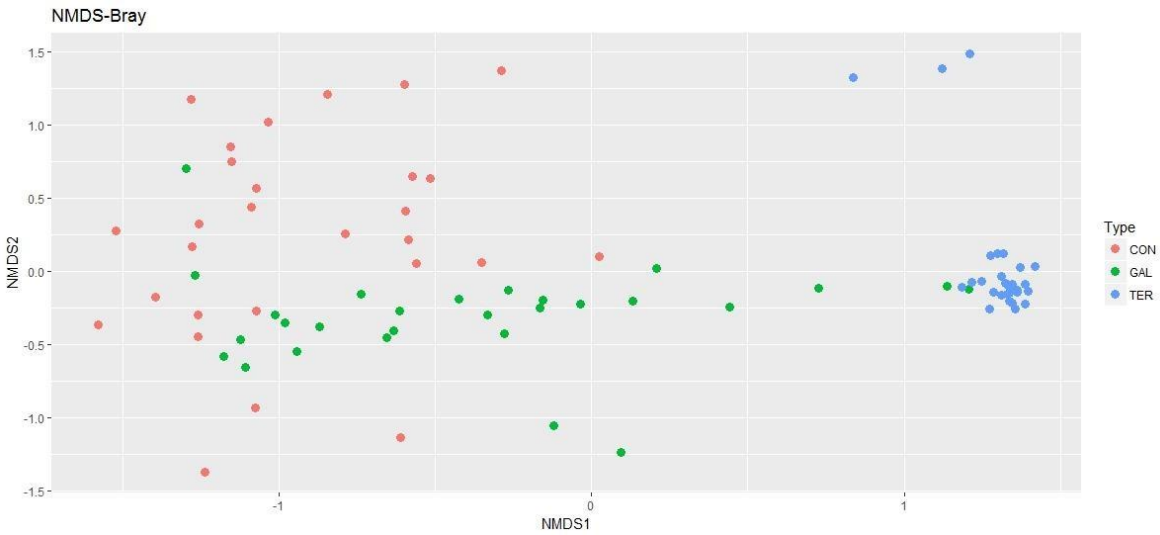


*Figure 12: **Non-metric multidimensional scaling** of the bacterial composition based on the termite samples. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies*

The overall results show that particular groups are not perfectly separated, what is not surprising taking into account that termites live in their environment. At the moment, my supervisor's team is processing samples of other termite species, and as part of them was collected in the same logs as my samples, the interspecific comparisons will hopefully bring the evidence of communities fixed to particular wood items vs. those spread by termites. The NMDS analysis on bacterial composition indeed showed the strong grouping pattern within the samples of termites, which are in general by far more similar to each other compared to any other wood samples, including those collected within the same trunk. This shows a high proportion of vertically transmitted symbiotic bacteria, which are often found in the termite galleries, likely from the faeces, what corresponds with the results of previous study (Su et al., 2016).

It seems that there is a gradient of bacterial composition from termites across galleries towards the control wood. Controls are more dispersed across the matrix, probably because the control wood is not affected by termites, but only by environmental factors over the time. Unfortunately, there are no data about the duration of the decomposition process, which could have effect on the microbial composition. My supervisor also disposes a sound wood sample from each of studied trunks, but establishment of the tree identity using molecular markers is only in progress now, leaving another important factor, i.e. the taxonomic position of the food source unavailable.

The NMDS analysis comparing the trunks among each other (see Figure 13) explains part of the differences observed among the samples. The termite display a relative stable position in the matrix. The position of galleries and control samples is variable, but still the position is closer between control samples and galleries, than between a food source and termites. The galleries which are in the matrix near to the termites are probably from old colonies where the galleries are changing a composition due to termite activity.

The same results show with PCoA analysis (Figure 14). Its show the absolute distances between types of samples. The termites are clearly forming a line-cluster which shows high similarity. With the termite samples near to the food source I assume a contamination from the food source, wood particles on their body or in the digestive tract.

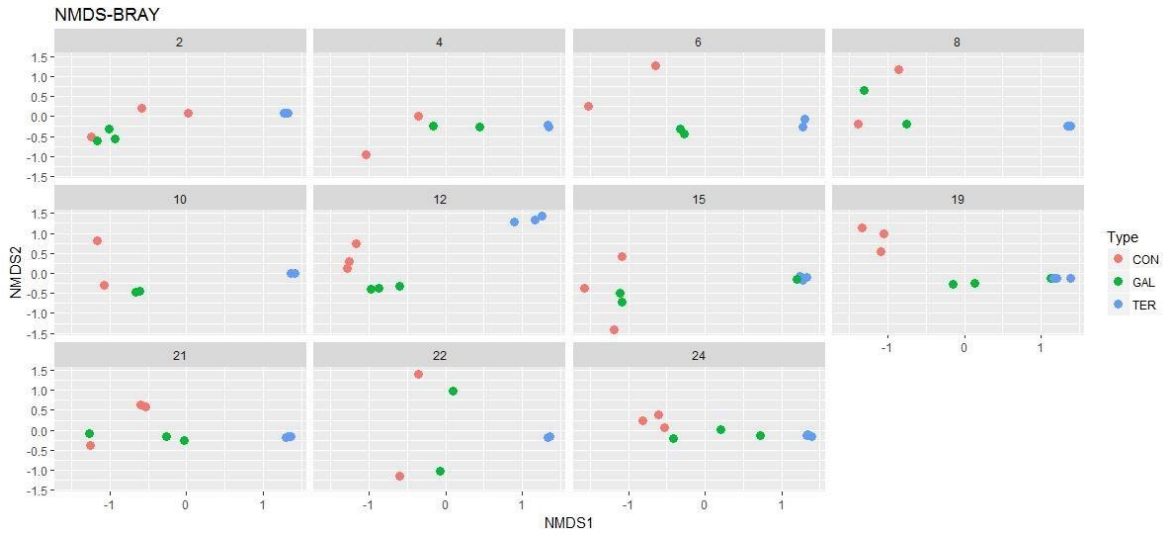


*Figure 13:* ***Non-metric multidimensional scaling*** *of the bacterial composition based on the termite samples shown on the tree trunks. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

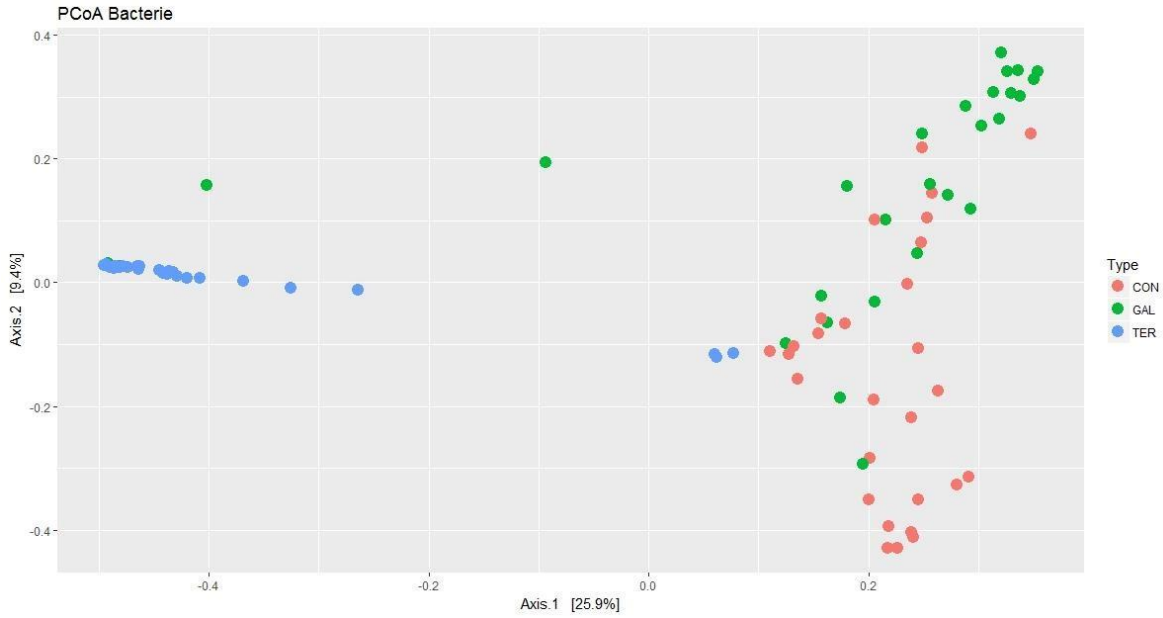


*Figure 14:* ***PCoA*** *analysis of bacterial composition based on termite samples. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

Control wood samples and galleries samples are showing on a Asis2 9.4 % variability. This variability relative low. Samples are mingled together but still we can distinguish samples with a higher variability, which are forming clusters on the edges of the matrix.

## 4.2. Results and discussion for fungal community

Apart of the Macrotermitinae having obligate and intimate relationships with their fungal associates (Aanen et al., 2002; Poulsen et al., 2014; Otani et al., 2016), the ectosymbiotic relationships between termites and fungi have barely been studied.

From the obtained 686016 sequences was obtained 45 OTUs with 3 % divergence rate of fungal ITS2. Out of these OTUs, 3 classified phyla of fungi were identified, one unclassified fungal cluster "UnclassF" 8 and four OTU completely unidentified according to ITS2 sequence. The relative abundance across the samples (See Figure 16) showed decreasing trend towards the termite samples with few exceptions. One of the exceptions is unclassified cluster OTU3, which is the most abundant cluster in the termites, virtually absenting in all other samples. OTU1, 2 and 4 seem to decrease their abundances from termite through galleries towards a control wood, where the specific reads occur considerably less frequently compared to the termite samples. OTU3 was moreover present only in the termites.

From the Figure 15 we can see the dominance of Basidiomycota and Ascomycota. In most of the samples of control wood the Basidiomycota is predominant phyla. The galleries display a mixture of different groups where the OTU1; OTU2 and OTU4 seems to be provide by termites where it belongs to the dominant groups. Relatively dominant but only in a single colony is OTU3. With no identification for that OTU3 cluster we cannot tell the reason of that high abundance within a single colony in termite samples.

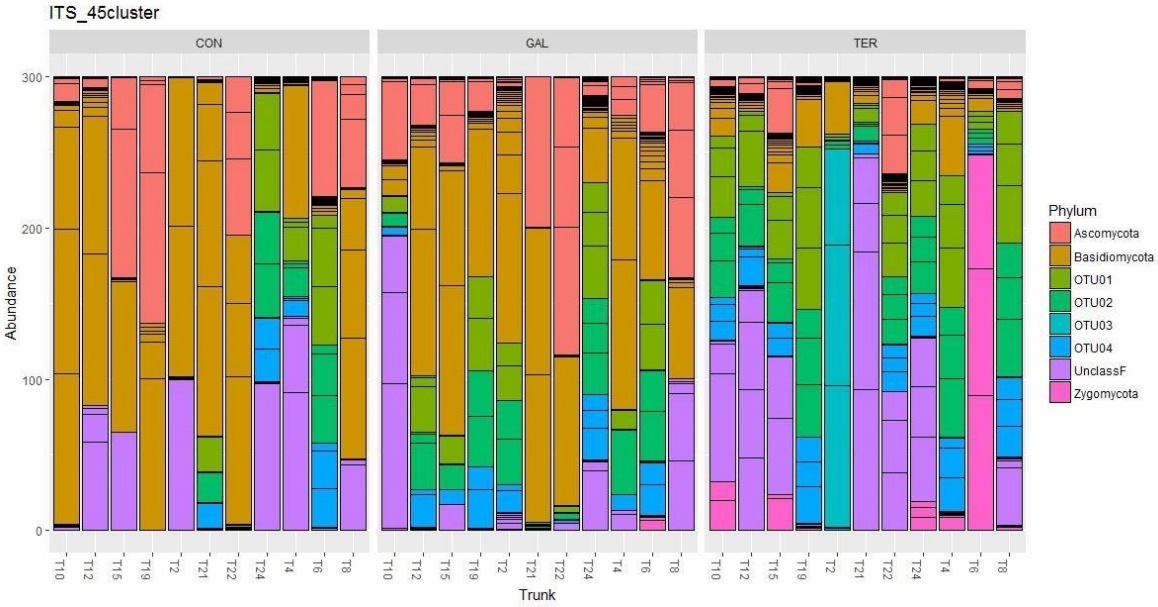


*Figure 15: Fungal relative abundancies within sampled trunks for each type of sample. Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

The unique fungal phyla found in the termite species belongs to genus *Umbelopsis* (Zygomycota: Mucorales). This fungus is known to occur with the wood feeding insects (Šrůtka, 2006), so is a good candidate for future studies as it can reveal some kind of association with the termite. Zygomycota seem to be a wide spread fungal taxon as they are shared in the 6 termite's colonies (Out of 11) The trunk with highest abundance of Zygomycota also show the transfer of this Fungal phylum in to the galleries.

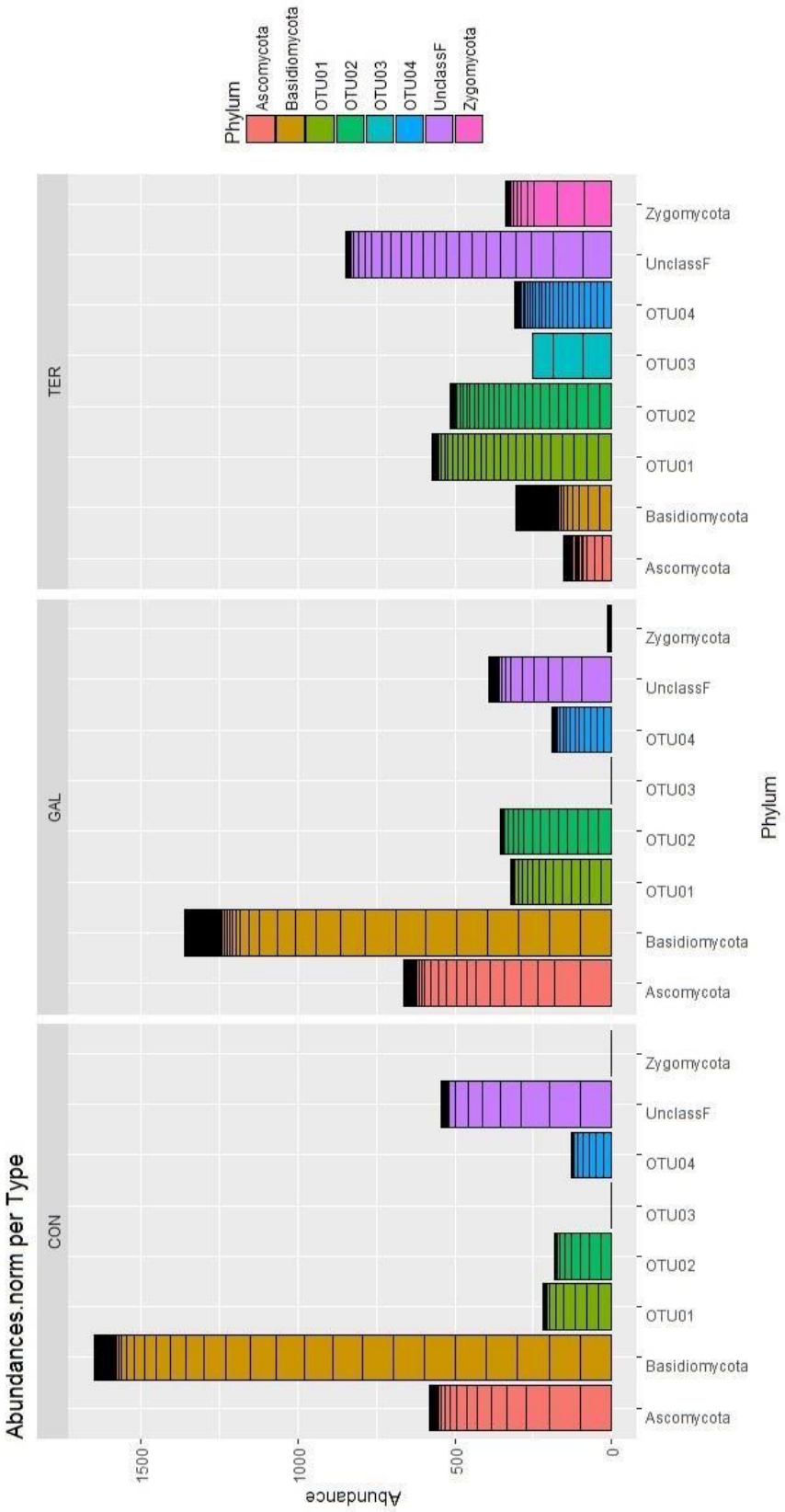


*Figure 16: Relative fungal phyla abundances shown for each type of samples.*

### 4.2.1 NMDS analysis for ITS

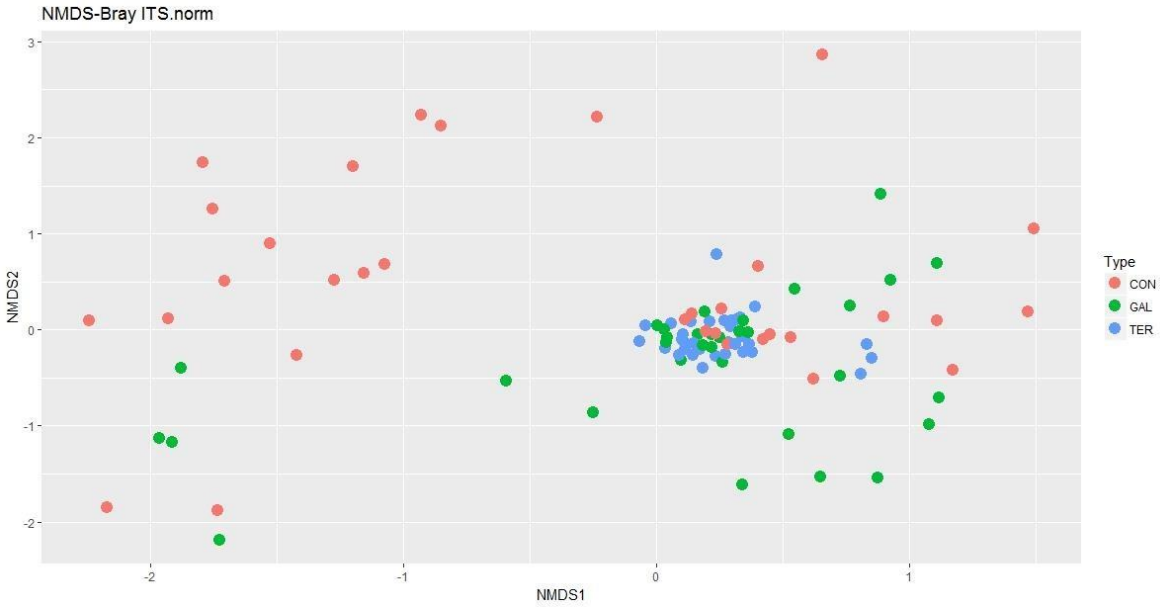


*Figure 17:* ***Non-metric multidimensional scaling*** *with normalised fungal abundances based on type of the sample (normalized data). Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

Established sample grouping by OTUs similarities displayed by NMDS analysis show a pattern specific to the kind of samples. Termites form a compact cluster, while galleries and controls are more dispersed in the matrix. The closer control wood samples are probably affected by environmental conditions and I assume that the colony was relatively old and wood was in higher stages of decomposition, as evidenced by occurrence of higher numbers of fungal OTUs. On the other hand, some of the more distant galleries samples from that mixed cluster are showing a similarity to the control wood. That variability between galleries samples are probably caused by a duration of termite activity.

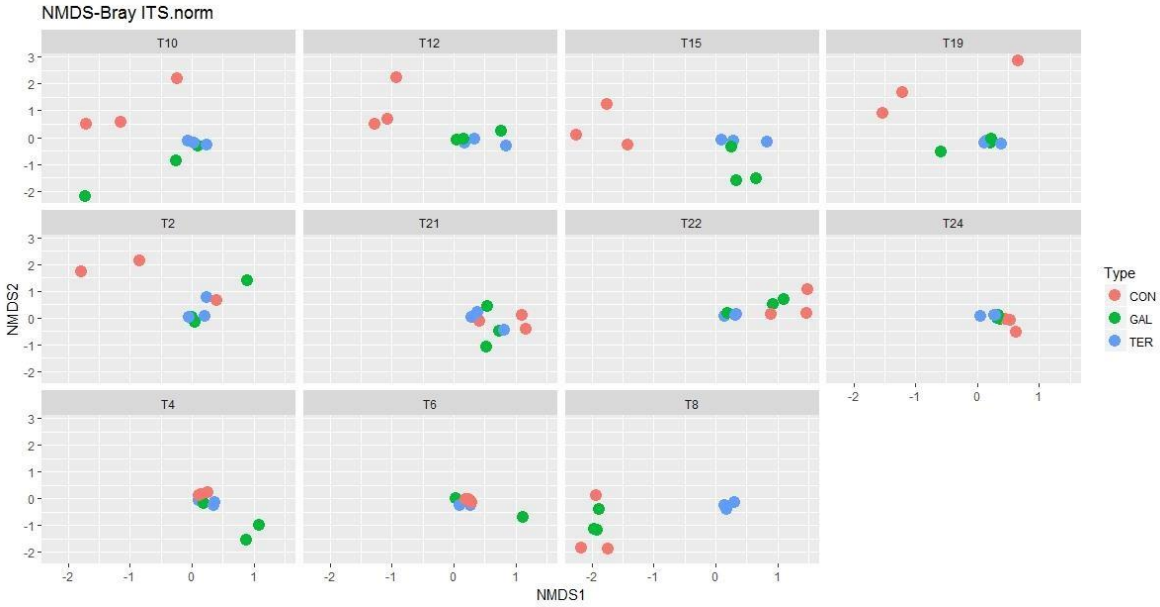


*Figure 18:* ***Non-metric multidimensional scaling*** *with normalised fungal abundances based on the type samples for individual trunks (T). Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

Figure 18 NMDS show the relative data for each trunk sampled. The pattern of fungal distribution varies across the samples. Probably in newly set colonies we can recognize the distances between termite samples and food source. The older trunks and colonies are forming a closer group. As we dispose the set of reference samples in 80 % alcohol, the age of colonies will be tested using a known phenomenon that in incipient colonies of Termitidae, the workers and soldiers tend to be smaller in body size compared to mature ones (Roisin, 2000). The PCoA (see Figure 19) shows the absolute abundances of fungi across all sampled trunks. In a matrix, we can again see most of the termite samples are relatively close forming compact units and the main variables are galleries samples and control wood. Overlapping galleries with a control wood display a very low variability between these two environments in fungal composition.

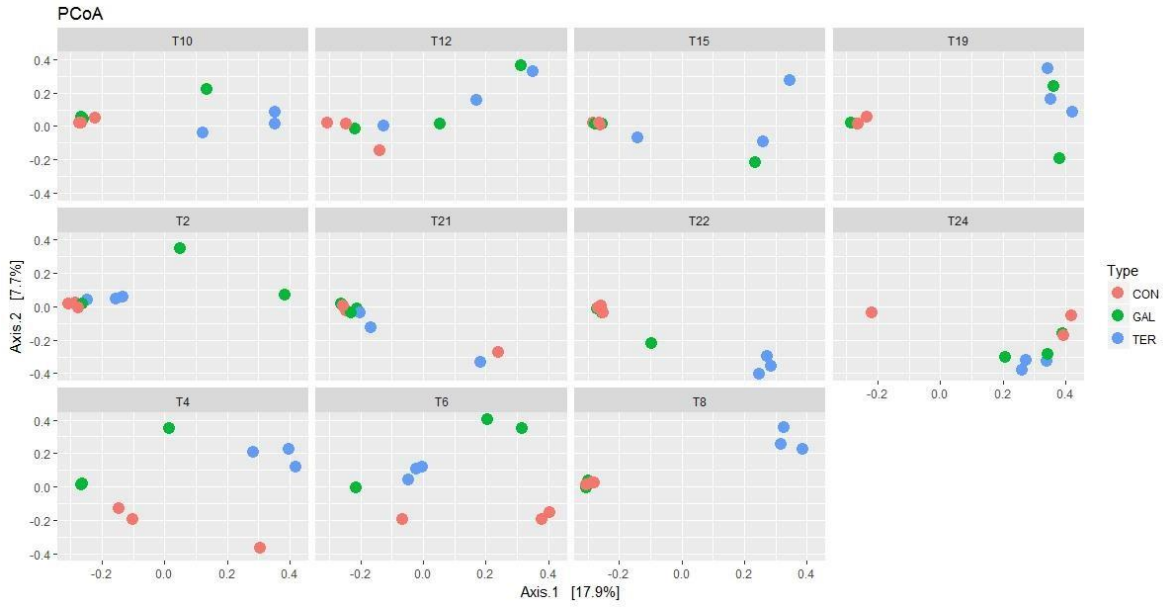


*Figure 19**: PCoA** analysis with absolute fungal abundances based on the type samples for individual trunks (T). Abbreviations: CON, control to food source; GAL, gallery = food source; TER, termite bodies.*

## 5. Conclusion

With this study, we put another small piece of work to a gigantic mosaic called termite associations with symbiotic microorganisms. To do so, we used *Nasutitermes octopilis* as a model species of wood-feeding termite common in Amazonia. Our results on bacterial composition shows the relative stability of the symbiotic associations, similarly to previous studies on the similar topic. At the same time, termites often contain the bacteria gathered from their environment, of which some may reach higher abundances in termite bodies compared to others. This can be seen as the first step in establishing the mutually profitable symbiosis. However, the importance of both symbiotic patterns to each other depend on the share of biochemical pathways shared together, a process which can take place for geological ages. At the same time, understanding the patterns of co-occurrence of microorganisms even in such a simple system will take much longer time and effort, what couldn't be done within this Master project from various reasons. I am anyway willing to finish these analyses as soon as possible.

The composition of fungal communities can vary and its dependent probably also on the environmental variables. Our result show an evidence of interaction, which represent a great opportunity for future studies, where we can focus deeper on these particular OTUs, supposed from having closer associations with our model termite species.

The main limitation of my work is a general lack of data on unique fungal or bacterial taxa in a reference database we used. We will surely continue on this topic with the aim to be able to distinguish between termite-associated and environmental taxa of fungi and bacteria. This work is among the first steps, we did on this way.

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