JOSÉ AUGUSTO MARTINS ROXINOL

THE MULTIPLE USE OF FECES BY MOUND TERMITES (BLATTODEA: ISOPTERA)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae.*

Orientador: Og Francisco Fonseca de Souza

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Orientador

Dedicatória

Para Ana Lígia, José †, Stela e Eduardo.

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Este trabalho é fruto de muita parceria. Agradeço ao CNPq (Processo: 142077–2016), à Universidade Federal de Viçosa, ao time do Departamento de Entomologia, do Programa de Pós-graduação em Entomologia, do laboratório de Termitologia e ao orientador pelas condições e recursos que me propiciaram para esta formação.

Cheguei aqui com muitas ideias e metas. Queria investigar coisas únicas, obter um destaque positivo como pesquisador, participar de eventos importantes, enfim, fazer algo para mudar as coisas. Só que a vida faz planos que nem sempre dão no que a gente espera. E aprendi que não há problema nenhum nisso. Pois, o mais valioso não é o que você procura, mas o que você acaba encontrando. O que importa nessa jornada são os companheiros de viagem. E nisso, eu fui um afortunado. Se hoje eu sorrio com orgulho, não é só pelos meus êxitos e pelo o que eu aprendi nessa caminhada, mas por vocês, meus companheiros. Por tê-los encontrado e por tê-los ao meu lado. Por isso, de forma bem singela deixo o registro de uma gratidão eterna a cada um de vocês. À minha família e amigos de outrora também o meu carinho.

Como o lapso do momento me fará omitir alguns nomes nestas linhas, eu não vou por nenhum nome em específico. Mas saibam que é no meu coração que cada um deles estão verdadeiramente registrados.

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Epígrafe

Shit happens!
(David Waltner-Toews, 2013)

Resumo

ROXINOL, José Augusto Martins, D.Sc., Universidade Federal de Viçosa, outubro de 2020. **Os múltiplos usos das fezes pelos cupins (Blattodea: Isoptera)**. Orientador: Og Francisco Fonseca de Souza.

As fezes são notoriamente um tópico desagradável para a maioria das pessoas. Entretanto, elas estão presentes no dia a dia de todos os animais e exercem funções muito além da excreção. Embora existam diversos exemplos de uso das fezes pelos animais, raros são os casos em que esses usos envolvam as fezes de conspecíficos. Isso porque estas fezes podem representar um alto risco de infecção de patógenos, já que nelas, patógenos podem se desenvolver. Apresentamos neste trabalho uma revisão investigando os usos das fezes pelos animais e em específico o caso dos cupins que são um dos raros grupos de animais que passam a vida em contato direto com a próprias fezes. O uso das fezes pelos cupins vale-se principalmente devido a ação dos microorganismos ali residentes, e foi se incrementando ao longo da evolução das linhagens de cupins. Tamanha relevância dessa massa fecal presente nos ninhos dos cupins, que ela é atualmente considerada a resistência estendida à doenças desses indivíduos, como já demonstrado para *Coptotermes formosanus* por Chouvenc *et al.* (2013). No interior dos ninhos dessa espécie há uma massa composta por fezes e madeira triturada, onde vivem micróbios que agem no controle de patógenos dos cupins. Aqui, apresentamos também um trabalho empírico onde investigamos os mecanismos por trás desta resistência estendida. Verificamos se os patógenos poderiam ser impedidos de se desenvolver (i) por efeito de exclusão competitiva promovida pelos microorganismos benéficos, ou (ii) por um efeito de supressão direta oriundo de substâncias presentes na massa fecal, sejam estas substâncias liberados pelos microorganismos benéficos ou pelo metabolismo dos próprios cupins. Usamos a massa fecal de ninhos *Cornitermes cumulans* para avaliar seu efeito na sobrevivência desses cupins expostos à infecção de um fungo patogênico. Além disso, usamos essa massa fecal com e sem a supressão de microorganismos para investigar se eles afetam a germinação do termitopatógeno. Verificamos que a massa fecal não impede a infecção patogênica e consequente surgimento de doenças no corpo dos cupins, mas diminui a reprodução do patógeno. E esse efeito é devido nã somente aos microorganismos da massa fecal, mas também à massa fecal em si. Assim, esses resultados demonstram que a massa fecal de alguns cupins não atua aumentando a resistência a doenças diretamente nos indivíduos, mas pode contribuir para sua saúde geral. Mais que isso, a massa fecal prejudica o desenvolvimento dos patógenos no ninho, atuando assim como uma agente profilático para a colônia, mesmo se os microorganismos benéficos nela residentes forem eliminados.

Palavras-chave: Entomopatógeno. Infecção. Insetos. Microorganismos. Solo.

Abstract

ROXINOL, José Augusto Martins, D.Sc., Universidade Federal de Viçosa, October, 2020. **The multiple use of feces by mound termites (Blattodea: Isoptera)**. Advisor: Og Francisco Fonseca de Souza.

Feces are notoriously an unpleasant topic for most people. However, they are present in the daily lives of all animals and perform functions far beyond excretion. Although there are several examples of the use of feces by animals, there are rare cases in which these uses involve conspecific feces. It is because these feces can represent a high risk of infection, since pathogens can grow on them. We present here a review investigating the uses of feces by animals and especially for the case of termites, which are one of the rare groups of animals that spend their lives in direct contact with their own feces. We found that the use of feces by termites is mainly due to the action of microorganisms residing there, and it was increased over the evolution of termites lineages. Such is the relevance of the fecal mass in termite nest, that it is currently considered to pose extended resistance to diseases, as already demonstrated for *C. formosanus* by Chouvenc *et al.* (2013). Inside the nests of this species, there is a mass composed of feces and crushed wood, where live the microbes that control pathogens of termites. Here, we present an empirical work where we investigate the mechanisms behind such an extended disease resistance. We verified whether pathogens would be impaired (i) by competitive exclusion from beneficial microorganisms, or (ii) by direct supression originated from substances present in the fecal mass, be those liberated from microorganisms or from termite metabolism itself. We used the fecal mass of nests *C. cumulans* to evaluate its effect on the survival of these termites exposed to fugal pathogenic infection. Besides, we use this fecal mass with and without the suppression of microorganisms to investigate whether they affect the germination of termitopathogens. We found that the fecal mass does not prevent pathogenic infection and the consequent emergence of diseases in the body of termites, but it reduces the reproduction of the pathogen. And this effect is not only due to the microorganisms in the fecal mass, but also to the some yet unknown component of the fecal mass. Thus, these results demonstrate that the fecal mass of some termites does not act to increase resistance to disease in individuals themselves, but it can contribute to their overall health. Moreover, it impairs the development of pathogens in the nest, thus acting as a prophylactic agent for the colony, even if the beneficial microorganisms therein resident would be elliminated.

Keywords: Entomopathogen. Infection. Insects. Microorganisms. Soil.

Sumário

1

Introdução Geral

As fezes são uma exclusividade dos indivíduos do reino animal. Embora ela possa causar repulsa na maioria de nós humano, é inegável que ela está presente diariamente em nossas vidas, e de outros animais (Waltner-Toews, 2013). Há quem as classifique com um mero dejeto do tubo digestivo (Dethlefsen et al., 2006; Kim et al., 2011; Rose et al., 2015). Entretanto, as fezes desempenham papeis que vão muito além da participação no processo digestivo. Muitos animais utilizam as fezes em atividades como comunicação, construções, defesa a inimigos, e até mesmo na alimentação (King *et al.*, 2016; Mizumoto and Bourguignon, 2020; Hogstad, 2004; Körner *et al.*, 2016; Diehl *et al.*, 2015).

Ainda que as fezes tenham utilidade para muitos animais, é raro encontrarmos indivíduos que entrem em contato com as fezes de conspecíficos (Weiss, 2006). Isso porque quando as fezes são deixadas expostas ao ambiente, elas se tornam alvo de micróbios potencialmente patogênicos (Blake *et al.*, 1980; Gilbert, 1997; Ibáñez-Álamo *et al.*, 2017). Esses micróbios usam as fezes como um meio de cultura para seu desenvolvimento. Assim, os casos de animais que utilizam ou estão em contato direto com as fezes são em geral com fezes oriundas de heteropecíficos (Weiss, 2006).

Um dos únicos organismos que vive em meio às fezes de conspecíficos e parece não ter problemas com isso são os cupins (Engel, 2019). Os cupins são insetos sociais que vivem em ninhos feitos em grande parte de fezes dos próprios indivíduos que habitam o ninho (Grassé, 1958; Korb, 2008). Curiosamente, as espécies de cupins que possuem os maiores ninhos e que também são as mais especiosas, são as que mais utilizam e mantém contato com suas fezes (Noirot and Darlington, 2000; Bucek *et al.*, 2019; Engel, 2019). Ainda que haja a presença de patógenos presente em suas fezes e ninhos. Esse fato permanece como um paradoxo para a biologia.

Neste trabalho investigamos o papel da massa fecal dos ninhos de cupins.

Nossa pergunta principal é por que os cupins matêm contato direto com suas fezes dentro do ninho, ainda que elas possam abrigar patógenos? buscamos identificar os mecanismos pelos quais alguns animais, como os cupins, lidam intimamente com as fezes de conspecíficos sem terem problemas com isso. Para isso, dividimos este trabalho em dois capítulos. No primeiro capítulo (Cap. 2), apresentamos uma revisão sobre como acontece a utilização das fezes pelos animais, e em específico para o caso dos cupins. No segundo capítulo (Cap. 3), apresentamos um trabalho empírico testando o efeito da massa fecal dos ninhos de *C. cumulans* (Isoptera: Termitidae) na resistência dos cupins à doenças patogênicas e também o seu efeito direto na reprodução de patógenos dos cupins.

2

We need to talk about feces - the truth about the number two in animal societies

2.1 Abstract

Feces are present in the daily lives of animals. Far beyond metabolic, feces can play a role in a range of activities in our biology. It is relatively easy to find examples of animals using feces for of communication, construction, defense, and even food. Although feces are a versatile resource for animals, they rarely use their own feces to perform some of these functions. It is because feces are a growth medium for microbes that are more likely to be pathogenic to the fecal former. So the usual practice is to avoid oneself's feces. However, termites are a rare animal group that live in close contact with their own during all life, without concerns about it. Here we review the main implications and limitations from feces usage by animals. In the end, we attempt to figure out the mechanisms by which some animals, such as termites, deal so intimately with feces without having problems with pathogens. In the particular case of termites, we found that the symbiotic association with microbes may favor feces usage. This may explain the reasons for the increase in feces usage over the evolution of termites lineages.

Keywords: Evolution. Fecal. Termites. Pathogens. Symbiosis.

2.2 Introduction

The feces, uniqueness from the animal kingdom, are the outcome of each individuals' digestive process. It is through feces, that individuals discharge food residuals, water, metabolites, chemical volatiles, and many microbes (Dethlefsen et al., 2006; Kim et al., 2011; Rose et al., 2015). But there are more things in the feces than just waste. The feces are an elaborated microenvironment that can bring us cues about how animal individuals and societies run their history, behavior, and health issues (Dunn et al., 2010; Waltner-Toews, 2013; Rodgers and Janečka, 2013; Dunn et al., 2013; Barberán et al., 2015). However, feces topics are usually unattractive for readers, who may be missing out a valuable source of information about their biology (Lewin, 2001; Fitzpatrick et al., 2013; McDonald et al., 2018).

Although we humans have a natural disgust for feces (Curtis et al., 2011), many animals do not. Animals can use feces to communicate, to build dwellings and nests, as a defense, to feed, or even so to improve their immunity (King et al., 2016; Mizumoto and Bourguignon, 2020; Hogstad, 2004; Körner et al., 2016; Holter, 2016; Diehl et al., 2015) . In spite of that, hardly any of these animals eat or live in their feces their whole life, because it can also bring them health problems (Weiss, 2006; Dunn et al., 2010; Walsh et al., 2013; Ibáñez-Álamo et al., 2017). After all, as a rule, feces are a waste and a risk to animals' health (Rose et al., 2015). When the feces are left in the environment, it becomes a target of ill-fated organisms that establishes and pullulates within it (Foster et al., 2013). These organisms often include pathogens, who can bring some harm or disease to the feces former (Blake et al., 1980; Gilbert, 1997; Ibáñez-Álamo et al., 2017). So, what is usual is that individuals adopt hygienic behaviors and manage feces far away from where they feed and sleep, avoiding any risk to their health (Weiss, 2006; Hart and Ratnieks, 2002; Sato et al., 2003; Rose et al., 2015; Ibáñez-Álamo et al., 2017).

Since the feces pose diseases' risk to individuals, animal societies usually concern with fecal accumulation near where they live (Sato et al., 2003; Lucas et al., 2019; Farji-Brener et al., 2016). The more individuals, the more feces production and accumulation, the more likelihood of pathogens' build up and consequential risk of disease, and so the more the society' need to concern about feces handling (Côté and Poulinb, 1995; Rifkin et al., 2012; Medina-Medina et al., 2014; Czaczkes et al., 2015; Mukhtar et al., 2016).

However, there is a particular case of an animal society that is always in touch with their feces without any problem. These are the termites. Termites are truly social societies that live as a coercive colony inside of a nest (Grassé, 1958; Korb, 2008). All types of termites' nests have, if not at all, at least one part composed by feces (Lee and Wood, 1971; Mizumoto and Bourguignon, 2020; Engel, 2019). In such nests, termites manipulate, walk on, and occasionally eat these feces (Eggleton, 2010; Korb, 2010). These behaviors are more common in the newest termites lineages, which build the biggest colonies and comprise the $\cong 70\%$ of termite species (Noirot and Darlington, 2000; Bucek et al., 2019; Engel, 2019). That is to say, intimate contact with large amounts of feces seems to be a derived trait that is a rule for the vast majority of termite species.

Therefore, living in the feces is not a concern to termites societies, even though it seems a paradox in biology. Why does it happen? Why was this behavior spread throughout termites' lineages? Herein, we review the literature on the feces under a social context. We will attempt to figure-out mechanisms by some animals, such as termites, deal so intimately with feces without having problems with pathogens.

In this review, we also suggest that termites feces usage is a derivative trait in Isoptera mediated by their symbiosis with the fecal microbes. Within these scenarios, we aim to discuss how these fecal microbes contributes with the fecal usage strategies on termites societies.

2.3 What are the feces?

Dung, stool, ordure and, an uncountable another way to say feces, is the exclusive product derived from animal digestion (Hill et al., 2012). If you've ever soiled your shoes on it, you probably don't want to talk about it. Perhaps, you've certainly had relief moments with yours, and so you should take a moment to learn more about it. Although we may ignore the feces, it is present and impactant for our daily life and for that of all other animals (Rowland, 1975). But, what are the feces, after all?

The feces are the solid or semisolid food residual mass egested by animals (Rowland, 1975; Hill et al., 2012; Waltner-Toews, 2013). Everything that is ingested and is not metabolized by an animal or by its internal guests is released in the fecal mass (Hill et al., 2012). In general, feces contain carbohydrates, inorganic materials, lipids, minerals, proteins, toxins, and water that are derived from the food digestion (Ervin et al., 2013; Rose et al., 2015; Penn et al., 2018). Besides such compounds, feces can hold a high diversity of hormones, toxins, and enzymes that arise from the metabolism of the individual or its gut symbionts (Paton and Paton, 1998; Hill et al., 2012; Rose et al., 2015). A high diversity of microbes can also be excreted in feces. This diversity of elements make feces a gem cue to screaning the individual's ecology and health (Putman, 1984; Fitzpatrick et al., 2013; Barberán et al., 2015). All in all, the individuals are not what they eat, they are what they excrete.

2.4 The feces role in the animal kingdom

The feces are daily present in animal lives (Rowland, 1975). Although it seems to be just a waste of the digestive process, the feces are used by many animals also for communication (Kappeler, 1990), buildings (Lee and Wood, 1971), defense (Hogstad, 2004), nutrition (Nichols et al., 2008), or even to improve their immune system (Diehl et al., 2015). In this section we review these feces usage in the animal kingdom.

2.4.1 Communication

Many animals use feces' odour to communicate and share information with other individuals about territory occupancy, mate availability, group status, or immune conditions (Roper et al., 1993; Jordan et al., 2007; Dröscher and Kappeler, 2014; Eppley et al., 2016). The feces' odor marks are advantageous to remain active for a long time even when the marker is absent, and also is a honest and cheat-proof information source from another individual (Zala et al., 2004; Ruibal et al., 2010; Piñeiro and Barja, 2012).

By defecating, individuals can mark their territory to avoid intruders. Due this effect, many animals select especific places to defecate. For example, the European Wildcat spread out their feces on specific plants that maximize the dissipation of the odorous signal (Piñeiro and Barja, 2012). These marks can affect the spatial distribution of males preventing agonistic encounters. Similar, wolves, badgers, and lizards also use the feces to signal their territories, and to inform their presence in the area (Zub et al., 2003; Roper et al., 1993; Labra et al., 2002).

The feces can also inform the presence of a conspecific in the area, to indicate social status or even the immune and physiological conditions of a individual. Many species have sites where individuals defecate repeatedly, with the aim of group communication (Dröscher and Kappeler, 2014; Eppley et al., 2016). The White-footed Sportive Lemurs live in an uncoercive family group and each individual from the family group urinates and defecates in the same tree as if it was a family's latrine. Such a behavior reinforces the group bound relationship because the odor derived from their latrines informs the family members of how is the health of other relatives, even if they rarely meet (Dröscher and Kappeler, 2014). The latrines have been used by several animals since the middle-late Triassic (Fiorelli et al., 2013). On the other hand, it only started to be used in our species with the emergence of cities ($\simeq$ 3 mya) (Wald, 2016). This latrines are considered a mark of our civilization, manly due its sanitary importance (Antoniou et al., 2016; Wald, 2016).

Besides family latrines, there are communal latrines, which are bigger latrines shared by many conspecifics. These communal latrines are often a form of scent-marking behavior, hence a resource to olfactory communication and territorialism (Jordan et al., 2007; Eppley and Donati, 2011; King et al., 2016). For instance, Badgers, Raccoon Dogs, and Spotted Hyaena are true latrine users and share information from the groups or about their territory (Roper et al., 1993; Yamamoto, 1984; Mills and Gorman, 1987). Likewise, Meerkat groups share communal latrines with neighboring groups aiming to monitor each other by the odor, avoiding agonistic confrontations (Jordan et al., 2007).

While communal latrines are useful to inform territorial property of social species, solitary ones use communal latrines mainly to inform about mate availability, or to recognize non-specific and conspecific use of the area. For example, in the ocelot societies, latrines provide clues on the reproductive status of conspecifics while informing their presence to heterospecifics (usually preys) (King et al., 2016). Likewise, white rhinos can discriminate by the feces odor the territory of other males and, familiar or unfamiliar females, while the feces odor of house mice males indicates to females the chance of this male having any bacterial infection (Cinková and Policht, 2015; Walsh et al., 2013).

Accordingly, in invertebrates such as Triatominae bugs, coackroaches, and termites, to pose feces signals in latrine-like places can be helpfull to aggregate conspecifics (Nalepa et al., 2001; Wada-Katsumata et al., 2015; Mosquera and Lorenzo, 2020). This behavior is also conjectured as paving the origin of symbiotic mutualism with microorganisms in cockroaches and termites (Nalepa et al., 2001; Legendre et al., 2015; Evangelista et al., 2019; Nalepa, 2020). The aggregation runs because of aggregation' pheromones production in dictyopterans that drove from volatile carboxylic acids produced by their gut microbes community (Wada-Katsumata et al., 2015).

2.4.2 Building

While feces can be the animals' social media, being useful to share information, another peculiar way of feces usage is to build dwellings and nests. Such building method is used by many animals. In such animal buildings, the feces are used as an organic cement to structure the building materials (Vilane, 2010; Millogo et al., 2016).

The termites are arguably the engineers that are more reliant on feces for their buildings (Lee and Wood, 1971). Termite spread their feces in walls and galleries inside of the nest (Cosarinsky, 2011; Korb, 2010). Is thought that this feces can serve as a fertile medium to raise microbes that are beneficial to the termite colony (Chouvenc et al., 2013). Furtermore, termite feces provide moisture retention and cementing, which also contributes to the nest' micro-climate control (Wood, 1988). Some stingless bees can also build the external part of their nest using a mass composed of feces and other materials (Medina-Medina et al., 2014). Unlike termites, these bees keep the internal part of their nests feces-free.

Other animals can also use feces produced by heterospecifics. For instance, several ovenbirds in the family Furnariidae construct their nests adding dung to the clay mass (Zyskowski and Prum, 1999; Irestedt et al., 2006). In addition to its cementing properities, herbivores dung holds microbes and compounds which show antiseptic action and exert prophylaxis (Gupta et al., 2016). More close to us, our ancient cultures also employ cow dung in its home constructions (Gur-Arieh et al., 2019).

2.4.3 Defense

The feces also can be used as a cheap alternative for defense, against enemies or predators. Some human ancient civilizations are known to have made military use of feces against enemies (Eneh, 2012). The weapon-agents of these feces were putative pathogens carried by them, and their disgusting odors (Eneh, 2012). Likewise, some fieldfare birds eject their feces against predators that attempt to prey their eggs. Such behavior is a deterrent against avian predators because the feces' chemical compounds impregnate and erode the insulating properties of predator plumage (Hogstad, 2004).

Other defense strategies using feces are more passive, acting as camouflage or body shield. Some inquiline termites, for instance, defecate in front of their heterospecific termite hosts, thereby preventing their direct contact (Hugo et al., 2020). These strategies are not exclusive of little individuals however. Some sperm whales, for example, eliminated feces in the sea when feeling under threat. Such a behavior creates a fecal camouflage that avoids potential harassment from dolphins (Scott and Cordaro, 1987). In other cases, preys camouflage their position using their feces with other waste materials to build unattractive structures to predators. In case of orb-web spiders, these structures are stick-like (Tso, 1998; Gonzaga and Vasconcellos-Neto, 2005), or even like a bird dropping (Liu et al., 2015). On their turn, the larvae of the tortoise beetle uses their feces to construct body shield structures that hinder the predator's attack (Eisner and Eisner, 2000).

Several chrysomelid beetles cover their eggs with feces, and their larvae do it around themselves, both aiming to hide from predators (Eisner and Eisner, 2000). Besides acting as a physical barrier, these shield feces preset deterrent or repellent compounds that work against predators' onslaught (Vencl et al., 2009).

Shield-like structures made of feces are also employed by termites to avoiding pathogens build-up or to block the spread of odorous information. When a conspecific die, its nestmate termites workers bury their corpse in feces to avoid that microbes build-up and contaminate the nest (Chouvenc and Su, 2010; Chouvenc et al., 2012). Additionally, during confronts, termite soldiers usually spray chemical compounds to harm the the enemy. Because these compounds also serve to recrute other soldiers while driving workers away to safety. After such enemy is obliterated, workers cover it with feces to interrupt the chemical alarm signal transmission (Eisner et al., 1976).

2.4.4 Nutrition

Although it may sound strange, some individuals eat feces. Those individuals are called coprophage (Soave and Brand, 1991). Coprophagous animals may eat on feces derived from conspecifics or heterospecifics to obtain a dietary or immune intake (Ebino, 1993). Certainly the most famous coprophage is the dung beetle (Nichols et al., 2008). Dung beetles mostly feed on feces from mammalian herbivores (Holter and Scholtz, 2006). They extract nitrogen from feces microbial biomass and, essential amino acids and cholesterol from fecal content (Holter, 2016).

While dung beetles are truly coprophagous because feces is the main resource of their diet, other animals also eat feces but are not strict coprophagous (Soave and Brand, 1991). These animals occasionally feed on other' feces aiming to improve their nutritional demands (Fenolio et al., 2006). Such behavior is related as an adaptative feeding strategy to energy supply in food scarcity periods (Leuchtenberger et al., 2012). For example, during low food availability seasons, many not strict coprophage species of birds, reptiles, and mammals eat feces let by giant otters in their communal latrines. These animals eat the giant otters' feces because it present high nutrient and microbial content (Leuchtenberger et al., 2012).

It is also common to found some especies that eat their own feces or from conspecifics. In such a case the aim of this behavior is to recycle nutrients and chemical compounds excreted through feces (Hirakawa, 2008; Sakamaki, 2010). For instance, voles, leporids, and other mammalian herbivores eat their feces to extend the retention time of food in the gut and increase nutrient and vitamins intake (Ebino, 1993; Lee and Houston, 1993; Hirakawa, 2008). In some cases, youngs eat parent excrements to acquire microbes and enzymes (Osawa et al., 1993; Tobey et al., 2006).

Likewise, some dermapterans earwings keep their feces inside the nest during their initial growth period (Körner et al., 2016). Their juvenile relatives exchange feces between themselves, in some food deprivation moments. By decreasing these juveniles' need for food, this behaviour decreases their competition and aggressiveness, acting also as a drive for social cooperation (Körner et al., 2016). Similarly, piglets have their weigth and white blood cell increased if they are exposed to their mothers' feces in the nursery at the first days post-partum (Aviles-Rosa et al., 2019). The same happens to koalas youngs eat a substance called 'pap', that originates from their mother's caecum. The microbes and enzimes present in this pap improve the joey's ablility to digest eucalyptus leaves (Osawa et al., 1993; Tobey et al., 2006).

2.4.5 Pathogen resistance

Besides dietary enrichment, some species eat feces to improve their immune system (Diehl et al., 2015; Robertson et al., 2018). In such, neonates usually eat the parents' feces to increase the gut' microbes content (Hirakawa, 2008). Many of these microbes are symbiotic of these animal species and are vertically transmitted through generations (Nalepa et al., 2001; Jahnes et al., 2019; Nalepa, 2020). In rabbits, some symbiotic bacterias acquired from eaten feces, adhere to gut mucosa and impair the occurrence of other pathogenic bacterias (Combes et al., 2013). It is proposed that competitive exclusion and the inhibition by antimicrobial substances are mechanisms behind this symbiotic barrier effect (Combes et al., 2013).

Since the blatodeans ancestors, the termites' behavior of living in groups in dirty environments and surrounded by their feces has been maintained and increased (Nalepa et al., 2001; Nalepa, 2015, 2020; Engel, 2019). In this scenario, profitable microorganisms passed through the mesh of evolutionary pressures and were favored in this environment (Bucek et al., 2019). In contrast, these microorganisms were excellent competitors against termite pathogens (Zoberi and Grace, 1990; Roose-Amsaleg et al., 2004; Chouvenc et al., 2013; Sujada et al., 2014; Chouvenc et al., 2018). Thus the termites and these microorganisms coexisted, narrowing to the point of becoming symbiotics. Such a relationship allowed termites to increase the number of individuals in their colonies while they were able to increase the size of their nests, thus favoring more areas and feces, which are important for the development of microorganisms (Engel, 2019; Bucek et al., 2019). This symbiosis may have been one of the reasons for the reduction in the diversity and activity of immune genes as new termite lineages emerged (He et al., 2020).

The importance of fecal microbiota also is currently witnessed in the human medicine. Alternative therapies as fecal implant are employed attempting to increase diversity of microbes in human gut and alter the gut–brain communication, wich impact some gastrointestinal disorders, as colits (Giles et al., 2019; Wang et al., 2019; Wargo, 2020), and also many psychopathology and affective co–morbidities, such as depression, anxiety states, and cocoaine addiction (Dinan and Cryan, 2013; Kiraly et al., 2016; Xu et al., 2018). The efficiency of these therapies relies in restoring lost essential gut' symbionts. Such symbionts are the humans' old friends, that have long been helping humans to improve their pathogen resistance (Rook, 2010; Rook et al., 2014).

2.4.6 Diseases source

Althoug feces can be used as comunication, building, defense, nutrition, or pathogen control, feces as a disease source is one of the main concerns of animals (Putman, 1984; Sato et al., 2003; Livingston et al., 2005; Walsh et al., 2013; Waltner-Toews, 2013; Czaczkes et al., 2015; Farji-Brener et al., 2016; Ibáñez-Álamo et al., 2017; Lucas et al., 2019). When an animal defecates it brings a growth medium to microbes (Foster et al., 2013), which may pose pathogenic risks to those who enter in contact with these feces (Blake et al., 1980; Gilbert, 1997; Ibáñez-Álamo et al., 2017). Due to such risks, animal societies usually concern with fecal accumulation near where they live (Sato et al., 2003; Lucas et al., 2019; Farji-Brener et al., 2016).

2.5 The feces' use by termites in an eco-evolutive context. Why keep feces inside the home can be good?

The vast majority of animals which are able to profit from the use of feces do not live for all lifetime enclosured by their feces (Dunn et al., 2010; Walsh et al., 2013; Ibáñez-Álamo et al., 2017). In doing so, they avoid being exposed to pathogens longer than strictly needed to a healthy use of the feces (Blake et al., 1980; Gilbert, 1997; Foster et al., 2013; Ibáñez-Álamo et al., 2017). Termites, however, pose a notable exception to this rule, as they do spend all their lifetime surrounded and in continuous contact with their feces (Bignell, 2011).

The termites' behavior of feces' usage is derived from ancestral blattodeans that already used feces for comunication or protection against pathogens (Figure 2.1) (Nalepa et al., 2001; Wada-Katsumata et al., 2015; Rosengaus et al., 2013; Jahnes et al., 2019). Communication seems to be the first role for feces in blatodeans, appearing very early in their oldest family (Nalepa et al., 2001; Wada-Katsumata et al., 2015). Their feces contain a characteristic, odor produced by their gut microbes, that attracts and aggregates conspecifics (Nalepa et al., 2001; Wada-Katsumata et al., 2015). Later on, in the last cockroach family before termites, appear the use of feces as a source of pathogen control (Rosengaus et al., 2013).

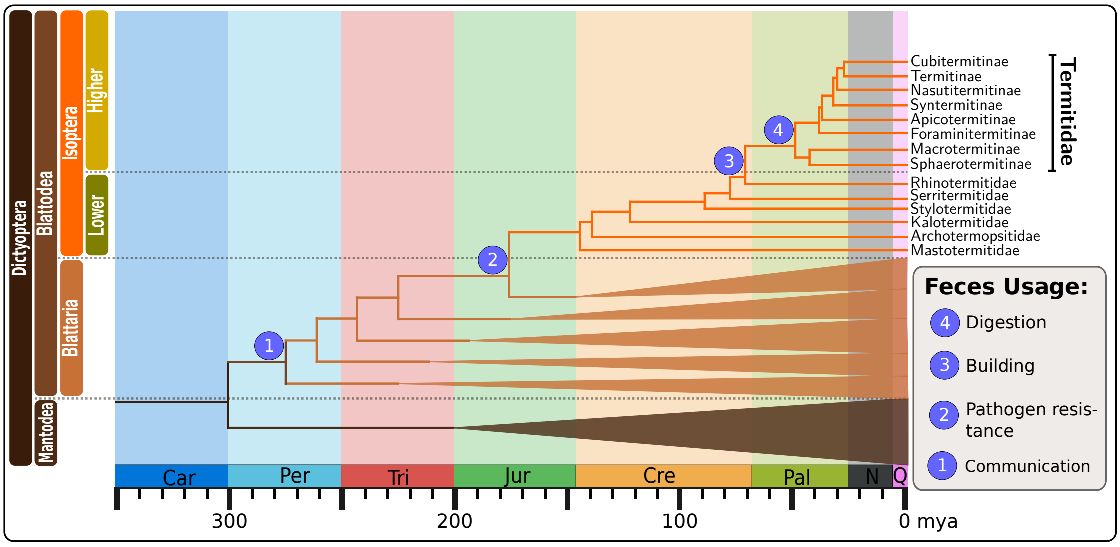

Figura 2.1: Evolution of feces usage by termites lineages.

After the cockroaches, raised the first isopteran species, called Lower Termites. These species form small colonies with dozens or hundreds of individuals that live in a wood nest (Bignell, 2011). These termites truly eat their home, which is a pure cellulose source. To diggest such cellulose, they are helped by endosymbiont flagellate protist microbes, that live in their gut and diggest the cellulosic fragments releasing glucose and other compounds necessary to termites' metabolic demands (Bignell, 2011; Nalepa, 2020).

The feces of these termites are rich in organic matter and microbes (Bignell, 2011). Most of them keep the produced feces inside the nest during all the colony lifetime, rather than isolate or release it outside of the nest, as does their cockroach ancestor and other social insects (Bignell, 2011; Medina-Medina et al., 2014; Wada-Katsumata et al., 2015; Czaczkes et al., 2015). It probably happens because, at least for some of these species, their feces contain compounds and microbes that act against pathogens infection (Rosengaus et al., 2013; Chouvenc et al., 2013; Peterson and Scharf, 2016).

Termites feces effectively impair pathogens to grow inside the nest, and hence it has a prophyllactic role (Rosengaus et al., 2013; Chouvenc et al., 2013; Peterson and Scharf, 2016). It is known that microbes found in termites' feces, nest, and gut, are passed from parents to siblings and also between colony residents, by proctodeal trophallaxis (Nalepa et al., 2001; Nalepa, 2015, 2020). This proctodeal trophallaxis is derived from ancestor blatodeans that are coprophagous and often display this behavior (Nalepa et al., 2001; Wada-Katsumata et al., 2015).

A important shift in the termite lineages occured in the last step of Lower Termites, with the emergence of *Coptotermes* group from the Rhinotermitidae family. Since Coptotermes group, termites conquered soil niches, and with it there was a shift in their nest pattern, from wood to soil made (Bignell, 2011; Chouvenc et al., 2013; Engel, 2019). As soils are dirtier environments than woods and have more microbe diversity, including entomopathogens, the exploration of soil niches propitiates the acquision of pathogens (Milner et al., 1998; Bruck, 2009; Sujada et al., 2014).

After Rhinotermitidae family, the Isoptera radiation raised their biggest group, the Higher Termites, which comprise the single-family Termitidae. Puzzling as it may sound, the Termitidae not only explore such a risky environment but they also form the most speciose and abundant family in the Isoptera (Bignell, 2011; Engel, 2019). Their normally build nests which house thousand to millions of individuals and can attain sizes that are enourmous even from a vertebrate-scale perspective (Bucek et al., 2019; Engel, 2019; Mizumoto and Bourguignon, 2020).

If the issues related to living in dirtier environments were not enough, these big colonies also accumulate their feces within their premises. While certainly a problem for any other animal society, these feces seem to not bother the Termitidae at all. The Termitidae individuals use their feces to construct the nest, mixing them with clay soil, saliva, and organic matter (Mizumoto and Bourguignon, 2020). These feces have cement proprieties that, after maturation keep the nest hard (Wood, 1988).

The nest of Termitidae species present a high amount of fecal mass and microbes in dedicated locals or even spread through galleries (Bignell, 2011). The microbes from this dirty nest part are composed by symbiotic microbes but also some pathogenic microbes. Oddly, such locals also shelter the termite juveniles forms, who have their immune system still in development (Grassé, 1958; Traniello et al., 2002). Since also juveniles are in direct contact with the potentially pathogenic microbes in addition to the symbionts, they are the colony individuals most prone to pathogenic damage. Due to this, is suspected that the feces and symbionts microbes act as a nests' firewall against termitopathogens infections, further plays the benefits previously mentioned here. This idea is proposed by the extended disease resistance hypothesis (Chouvenc et al., 2013).

The ectosymbionts' relevance as antipathogenics seems to be more probable in the recent termite' groups because they, unlike elder lineages, present decreased activity and diversity of their immune genes (He et al., 2020). Keeping the feces inside the nest may favor the growth of ectosymbiont microbes able to fight pathogenic ones. The success of the Isoptera seems hence tightly liked to a summation of fecal managing behaviors, since defecation by an ancestor cockroach that aggregated conspecifics until the peculiar behavior of keeping feces inside the home to favor nutritional demands and promote diseases defense. All these behaviors are notoriously mediated by microbes present in their feces.

2.6 Conclusion

Although talking about feces is usually a bit unpleasant or creates discomfort for most people, feces are part of the daily life of any animal, which includes us. Faeces are used by animals as a means of communication, construction, as a defense system, and even as food and a prophyllactic strategy. Here, we highlighted that termites are a rare group of animals that use feces in all these tasks, in addition to living permanently in contact with their own feces.

Evidence shows that the use of feces by termites derives from the first blatodeans as a form of communication and continued to be used as a way of arresting pathogens, in the construction of nests and finally, in food. In all these functions, the participation of symbiotic microbes is remarkable and indicates a profitable relationship for both parties, termites and microbes. So the usage of feces mediated by microbes may have far-reaching evolutionary consequences, at least to termites societies.

2.7 Bibliography

Antoniou, G. P., Feo, G. D., Fardin, F., Tamburrino, A., Khan, S., Tie, F., Reklaityte, I., Kanetaki, E., Zheng, X. Y., Mays, L. W., and Angelakis, A. N. (2016). Evolution of toilets worldwide through the millennia. *Sustainability*, 8(8):779.

Aviles-Rosa, E. O., Rakhshandeh, A., and McGlone, J. J. (2019). Preliminary study: depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. *Animals*, 9(5):268.

Barberán, A., Dunn, R. R., Reich, B. J., Pacifici, K., Laber, E. B., Menninger, H. L., Morton, J. M., Henley, J. B., Leff, J. W., Miller, S. L., and Fierer, N. (2015). The ecology of microscopic life in household dust. *Proceedings of the Royal Society B*, 282(1814):20151139.

Bignell, D. E. (2011). Morphology, physiology, biochemistry and functional design of the termite gut: an evolutionary wonderland. In Bignell, D. E., Roisin, Y., and Lo, N., editors, *Biology of Termites: a Modern Synthesis*, pages 375–412. Springer, Netherlands.

Blake, P. A., Weaver, R. E., and Hollis, D. G. (1980). Diseases of humans (other than cholera) caused by vibrios. *Annual Review of Microbiology*, 34:341–367.

Bruck, D. J. (2009). The ecology of fungal entomopathogens. *BioControl*, 55:103–112.

Bucek, A., Šobotník, J., He, S., Shi, M., McMahon, D. P., Holmes, E. C., Roisin, Y., Lo, N., and Bourguignon, T. (2019). Evolution of termite symbiosis informed by transcriptome-based phylogenies. *Current Biology*, 29(21):3728–3734.

Chouvenc, T., Efstathion, C. A., Elliott, M. L., and Su, N.-Y. (2013). Extended disease resistance emerging from the faecal nest of a subterranean termite. *Proceedings of the Royal Society B: Biological Sciences*, 280(1770):1– 9.

Chouvenc, T., Elliott, M. L., Šobotník, J., Efstathion, C. A., and Su, N.-Y. (2018). The termite fecal nest: A framework for the opportunistic acquisition of beneficial soil *Streptomyces* (Actinomycetales: Streptomycetaceae). *Environmental Entomology*, 47(6):1431–1439.

Chouvenc, T., Robert, A., Sémon, E., and Bordereau, C. (2012). Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. *Insectes Sociaux*, 59:119–125.

Chouvenc, T. and Su, N.-Y. (2010). Apparent synergy among defense mechanisms in subterranean termites (Rhinotermitidae) against epizootic events - the limits and potential for biological control. *Journal of Economic Entomology*, 103(4):1327–1337.

Cinková, I. and Policht, R. (2015). Discrimination of familiarity and sex from chemical cues in the dung by wild southern white rhinoceros. *Animal Cognition*, 18:385–392.

Combes, S., Fortun-Lamothe, L., Cauquil, and Gidenne, T. (2013). Engineering the rabbit digestive ecosystem to improve digestive health and efficacy. *Animal*, 7(9):1429–1439.

Cosarinsky, M. I. (2011). The nest growth of the neotropical mound-building termite, *Cornitermes cumulans*: A micromorphological analysis. *Journal of Insect Science*, 11(1):122.

Curtis, V., de Barra, M., and Aunger, R. (2011). Disgust as an adaptive system for disease avoidance behaviour. *Philosophical Transitions of the Royal Society B*, 366(1563):389–401.

Czaczkes, T. J., Heinze, J., and Ruther, J. (2015). Nest etiquette - where ants go when nature calls. *PLoS ONE*, 10(2):e0118376.

Côté, I. M. and Poulinb, R. (1995). Parasitism and group size in social animals: a meta-analysis. *Behavioral Ecology*, 6(2):159–165.

Dethlefsen, L., Eckburg, P. B., Bik, E. M., and Relman, D. A. (2006). Assembly of the human intestinal microbiota. *Trends in Ecology and Evolution*, 21(9):517–523.

Diehl, J. M. C., Körner, M., Pietsch, M., and Meunier, J. (2015). Feces production as a form of social immunity in an insect with facultative maternal care. *BMC Evolutionary Biology*, 15(40):1471–2148.

Dinan, T. G. and Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterology & Motility*, 25(9):713–719.

Dröscher, I. and Kappeler, P. M. (2014). Maintenance of familiarity and social bonding via communal latrine use in a solitary primate (*Lepilemur leucopus*). *Behavioral Ecology and Sociobiology*, 68:2043–2058.

Dunn, R. R., Davies, T. J., Harris, N. C., and Gavin4, M. C. (2010). Global drivers of human pathogen richness and prevalence. *Proccedings of the Royal Society B*, 277(1694):2587–2595.

Dunn, R. R., Fierer, N., Henley, J. B., Leff, J. W., and Menninger, H. L. (2013). Home life: factors structuring the bacterial diversity found within and between homes. *PLoS ONE*, 8(5):e64133.

Ebino, K. Y. (1993). Studies on coprophagy in experimental animals. *Experimental Animals*, 42(1):1–9.

Eggleton, P. (2010). An introduction to termites: biology, taxonomy and functional morphology. In *Biology of termites: a modern synthesis*, pages 1–26. Springer.

Eisner, T. and Eisner, M. (2000). Defensive use of a fecal thatch by a beetle larva (*Hemisphaerota cyanea*). *Proceedings of the National Academy of Sciences of the United States of America*, 97(6):2632–2636.

Eisner, T., Kriston, I., and Aneshansley, D. J. (1976). Defensive behavior of a termite (*Nasutitermes exitiosus*). *Behavioral Ecology and Sociobiology*, 1:83–125.

Eneh, O. C. (2012). Biological weapons-agents for life and environmental destruction. *Research Journal of Environmental Toxicology*, 6(3):65–87.

Engel, M. S. (2019). Termite evolution: a primal knock on wood or a hearty mouthful of dirt. *Current Biology*, 29(21):R1126–R1129.

Eppley, T. and Donati, G. (2011). Observations of terrestrial latrine behaviour by *Hapalemur meridionalis* in the Mandena littoral forest, southeast Madagascar. *Lemur News*, 15:51–54.

Eppley, T. M., Ganzhorn, J. U., and Donati, G. (2016). Latrine behaviour as a multimodal communicatory signal station in wild lemurs: the case of *Hapalemur meridionalis*. *Animal Behaviour*, 111:57–67.

Ervin, J. S., Russell, T. L., Layton, B. A., Yamahara, K. M., Wang, D., Sassoubre, L. M., Cao, Y., Sivaganesan, C. A. K. M., Holden, A. B. B. P. A., Weisberg, S. B., and Shanks, O. C. (2013). Characterization of fecal concentrations in human and other animal sources by physical, culture-based, and quantitative real-time PCR methods. *Water Research*, 47(18):6873–6882.

Evangelista, D. A., Wipfler, B., Béthoux, O., Donath, A., Fujita, M., Kohli, M. K., Legendre, F., Liu, S., Machida, R., Misof, B., Peters, R. S., Podsiadlowski, L., Rust, J., Schuette, K., Tollenaar, W., Ware, J. L., Wappler, T., Zhou, X., Meusemann, K., and Simon, S. (2019). An integrative phylogenomic approach illuminates the evolutionary history of cockroaches and termites (Blattodea). *Proceedings of the Royal Society B*, 286(1895):20182076.

Farji-Brener, A. G., Elizalde, L., Fernández-Marín, H., and Amador-Vargas, S. (2016). Social life and sanitary risks: evolutionary and current ecological conditions determine waste management in leaf-cutting ants. *Proceedings of the Royal Society B*, 283(1831):20160625.

Fenolio, D. B., Graening, G. O., Collier, B. A., and Stout, J. F. (2006). Coprophagy in a cave-adapted salamander: the importance of bat guano examined through nutritional and stable isotope analyses. *Proceedings of Royal Society B*, 273(1585):439–443.

Fiorelli, L. E., Ezcurra, M. D., Hechenleitner, E. M., Argañaraz, E., Taborda, J. R. A., Trotteyn, M. J., von Baczko, M. B., and Desojo, J. B. (2013). The oldest known communal latrines provide evidence of gregarism in triassic megaherbivores. *Scientific Reports*, 3(1):3348.

Fitzpatrick, M. C., Sanders, N. J., Normand, S., Svenning, J., Ferrier, S., , Gove, A. D., and Dunn, R. R. (2013). Environmental and historical imprints on beta diversity: insights from variation in rates of species turnover along gradients. *Proceedings of the Royal Society B*, 280(1768):20131201.

Foster, M. L., Dowd, S. E., Stephenson, C., Steiner, J. M., and Suchodolski, J. S. (2013). Characterization of the fungal microbiome (mycobiome) in fecal samples from dogs. *Veterinary Medicine International*, 2013:658373.

Gilbert, K. A. (1997). Red howling monkey use of specific defecation sites as a parasite avoidance strategy. *Animal Behaviour*, 54(2):451–455.

Giles, E. M., D'Adamo, G. L., and Forster, S. C. (2019). The future of faecal transplants. *Nature Reviews Microbiology*, 17:719.

Gonzaga, M. O. and Vasconcellos-Neto, J. (2005). Testing the functions of detritus stabilimenta in webs of *Cyclosa fililineata* and *Cyclosa morretes*

(Araneae: Araneidae): do they attract prey or reduce the risk of predation? *Ethology*, 111:479–491.

Grassé, P.-P. (1958). Sur le nid et la biologie de *Cornitermes cumulans* (Kollar), termites brésilién. *Insectes Sociaux*, 5(2):189–199.

Gupta, K. K., Aneja, K. R., and Rana, D. (2016). Current status of cow dung as a bioresource for sustainable development. *Bioresources and Bioprocessing*, 3:28.

Gur-Arieh, S., Madella, M., Lavi, N., and Friesem, D. E. (2019). Potentials and limitations for the identification of outdoor dung plasters in humid tropical environment: a geo-ethnoarchaeological case study from South India. *Archaeological and Anthropological Sciences*, 11:2683–2698.

Hart, A. G. and Ratnieks, F. L. W. (2002). Waste management in the leaf-cutting ant *Atta colombica*. *Behavioral Ecology*, 13(2):224–231.

He, S., Sieksmeyer, T., Che, Y., Mora, M. A. E., Stiblik, P., anjd Mark C. Harrison, R. B., Šobotník, J., Wang, Z., Johnston, P. R., and McMahon, D. P. (2020). Evidence for reduced immune gene diversity and activity during the evolution of termites. *bioRxiv*. doi: 10.1101/2020.07.09.192013.

Hill, R. W., Wyse, G. A., and Anderson, M. (2012). *Animal Physiology*. Sinauer Associates, Oxford, 3rd edition.

Hirakawa, H. (2008). Coprophagy in leporids and other mammalian herbivores. *Mammal Review*, 31(1):61–80.

Hogstad, O. (2004). Nest defence strategies in the fieldfare *Turdus pilaris*: The responses on an avian and a mammalian predator. *Ardea*, 92(1):79–84.

Holter, P. (2016). Herbivore dung as food for dung beetles: elementary coprology for entomologists. *Ecological Entomology*, 41:367–377.

Holter, P. and Scholtz, C. H. (2006). What do dung beetles eat? *Ecological Entomology*, 32(6):690–697.

Hugo, H., Cristaldo, P. F., and DeSouza, O. (2020). Nonaggressive behavior: A strategy employed by an obligate nest invader to avoid conflict with its host specie. *Ecology and Evolution*, 10(16):8741–8754.

Ibáñez-Álamo, J. D., Rubio, E., and Soler, J. J. (2017). Evolution of nestling faeces removal in avian phylogeny. *Animal Behaviour*, 124:1–5.

Irestedt, M., Fjeldså, J., and Ericson, P. G. P. (2006). Evolution of the ovenbird-woodcreeper assemblage (Aves: Furnariidae) - major shifts in nest architecture and adaptive radiation. *Journal of Avian Biology*, 37(3):260–272.

Jahnes, B. C., Herrmann, M., and Sabree, Z. L. (2019). Conspecific coprophagy stimulates normal development in a germ-free model invertebrate. *PeerJ*, 7:e6914.

Jordan, R., Cherry, M. I., and B.Manser, M. (2007). Latrine distribution and patterns of use by wild meerkats: implications for territory and mate defence. *Animal Behaviour*, 73(4):613–622.

Kappeler, P. M. (1990). Social status and scent-marking behaviour in *Lemur catta*. *Animal Behaviour*, 40(4):774–776.

Kim, M.-S., Park, E.-J., Roh, S. W., , and Bae, J.-W. (2011). Diversity and abundance of single-stranded DNA viruses in human feces. *Applied and Environmental Microbiology*, 77(22):8062–8070.

King, T. W., Salom-Pérez, R., Shipley, L. A., Quigley, H. B., and Thornton, D. H. (2016). Ocelot latrines: communication centers for neotropical mammals. *Journal of Mammalogy*, 98(1):106–113.

Kiraly, D. D., Walker, D. M., Calipari, E. S., Labonte, B., Issler, O., Pena, C. J., Ribeiro, E. A., Russo, S. J., and Nestler, E. J. (2016). Alterations of the host microbiome affect behavioral responses to cocaine. *Scientific Reports*, 6:35455.

Korb, J. (2008). The ecology of social evolution in termites. In *Ecology of social evolution*, pages 151–174. Springer-Verlag Berlin Heidelberg, Berlin.

Korb, J. (2010). Termite mound architecture, from function to construction. In *Biology of termites: a modern synthesis*, pages 349–373. Springer, Dordrecht.

Körner, M., Diehl, J. M. C., and Meunier, J. (2016). Growing up with feces: benefits of allo-coprophagy in families of the european earwig. *Behavioral Ecology*, 27(6):1775–1781.

Labra, A., Escobar, C. A., Aguilar, P. M., and Niemeyer, H. M. (2002). Sources of pheromones in the lizard *Liolaemus tenuis*. *Revista Chilena de Historia Natural*, 75(1):141–147.

Lee, K. E. and Wood, T. G. (1971). *Termites and soils*. Academic Press, London.

Lee, W. B. and Houston, D. C. (1993). The role of coprophagy in digestion in voles (*Microtus agrestis* and *Clethrionomys glareolus*). *Functional Ecology*, 7(4):427–432.

Legendre, F., Nel, A., Svenson, G. J., Robillard, T., Pellens, R., and Grandcolas, P. (2015). Phylogeny of dictyoptera: dating the origin of cockroaches, praying mantises and termites with molecular data and controlled fossil evidence. *Plos one*, 10(7):e0130127.

Leuchtenberger, C., Ribasa, C., Magnussona, W., and Mourão, G. (2012). To each his own taste: latrines of the giant otter as a food resource for vertebrates in Southern Pantanal, Brazil. *Studies on Neotropical Fauna and Environment*, 47(2):81–85.

Lewin, R. A. (2001). More on merde. *Perspectives in Biology and Medicine*, 44(4):594–607.

Liu, M.-H., Blamires, S. J., Liao, C.-P., and Tso, I. M. (2015). Evidence of bird dropping masquerading by a spider to avoid predators. *Scientific Reports*, 4:5058.

Livingston, T. R., Gipson, P. S., Ballard, W. B., Sanchez, D. M., and Krausman, P. R. (2005). Scat removal: a source of bias in feces-related studies. *Wildlife Society Bulletin*, 33(1):172–178.

Lucas, J. M., Madden, A. A., Penick, C. A., Epps, M. J., Marting, P. R., Stevens, J. L., Fergus, D. J., Dunn, R. R., and Meineke, E. K. (2019). *Azteca* ants maintain unique microbiomes across functionally distinct nest chambers. *Proceedings of the Royal Society B*, 286(1908):20191026.

McDonald, D., Hyde, E., Debelius, J. W., Morton, J. T., Gonzalez, A., Ackermann, G., Aksenov, A. A., Behsaz, B., Brennan, C., Chen, Y., DeRight Goldasich, L., Dorrestein, P. C., Dunn, R. R., Fahimipour, A. K., Gaffney, J., Gilbert, J. A., Gogul, G., Green, J. L., Hugenholtz, P., Humphrey, G., Huttenhower, C., Jackson, M. A., Janssen, S., Jeste, D. V., Jiang, L., Kelley, S. T., Knights, D., Kosciolek, T., Ladau, J., Leach, J., Marotz, C., Meleshko, D., Melnik, A. V., Metcalf, J. L., Mohimani, H., Montassier, E., Navas-Molina, J., Nguyen, T. T., Peddada, S., Pevzner, P., Pollard, K. S., Rahnavard, G., Robbins-Pianka, A., Sangwan, N., Shorenstein, J., Smarr, L., Song, S. J., Spector, T., Swafford, A. D., Thackray, V. G., Thompson, L. R., Tripathi, A., Vázquez-Baeza, Y., Vrbanac, A., Wischmeyer, P., Wolfe, E., Zhu, Q., , and Knight, R. (2018). American gut: an open platform for citizen science microbiome research. *mSystems*, 3(3):e00031–18.

Medina-Medina, L. A., Hart, A. G., and Ratnieks, F. L. (2014). Waste management in the stingless bee *Melipona beecheii* Bennett (Hymenoptera: Apidae). *Sociobiology*, 61(4):435–440.

Millogo, Y., Aubert, J.-E., Séré, A. D., Fabbri, A., and Morel, J.-C. (2016). Earth blocks stabilized by cow-dung. *Materials and Structures*, 49:4583–4594.

Mills, M. G. L. and Gorman, M. L. (1987). The scent-marking behaviour of the spotted hyaena *Crocuta crocuta* in the southern Kalahari. *Journal of Zoology*, 212(3):483–497.

Milner, R. J., Staples, J. A., Hartley, T. R., Lutton, G. G., Driver, F., and Watson, J. A. L. (1998). Occurrence of *Metarhizium anisopliae* in nests and feeding sites of australian termites. *Mycological Research*, 102(2):216–220.

Mizumoto, N. and Bourguignon, T. (2020). Modern termites inherited the potential of collective construction from their common ancestor. *Ecology and Evolution*, 10(13):6775 – 6784.

Mosquera, K. D. and Lorenzo, M. G. (2020). Triatomines of the genus *Rhodnius* do not mark shelters with feces. *Journal of Chemical Ecology*, 46(9):865–870.

Mukhtar, E. M., Williams, I. D., Shaw, P. J., and Ongondo, F. O. (2016). A tale of two cities: the emergence of urban waste systems in a developed and a developing city. *Recycling*, 1(2):254–270.

Nalepa, C., Bignell, D., and Bandi, C. (2001). Detritivory, coprophagy, and the evolution of digestive mutualisms in dictyoptera. *Insectes Sociaux*, 48:194–201.

Nalepa, C. A. (2015). Origin of termite eusociality: trophallaxis integrates the social, nutritional, and microbial environments. *Ecological Entomology*, 40(4):323–335.

Nalepa, C. A. (2020). Origin of mutualism between termites and flagellated gut protists: transition from horizontal to vertical transmission. *Frontiers in Ecology and Evolution*, 8(14).

Nichols, E., Spector, S., Louzada, J., Larsen, T., Amezquita, S., and Favila, M. (2008). Ecological functions and ecosystem services provided by Scarabaeinae dung beetles. *Biological Conservation*, 141(6):1461–1474.

Noirot, C. and Darlington, J. P. E. C. (2000). Termite nests: architecture, regulation and defence. In Abe, T., Bignell, D. E., and Higashi, M., editors, *Termites: Evolution, Sociality, Symbioses, Ecology*, pages 121–139. Kluwer Academic Press, Dordrecht.

Osawa, R., Blanshard, W., and Ocallaghan, P. (1993). Microbiological studies of the intestinal microflora of the koala, *Phascolarctos Cinereus* .ii. pap, a special maternal feces consumed by juvenile koalas. *Australian Journal of Zoology*, 41(6):611 – 620.

Paton, J. C. and Paton, A. W. (1998). Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews*, 11(3):450–479.

Penn, R., Ward, B. J., Strande, L., and Maurer, M. (2018). Review of synthetic human faeces and faecal sludge for sanitation and wastewater research. *Water Research*, 132:222–240.

Peterson, B. F. and Scharf, M. E. (2016). Lower termite associations with microbes: synergy, protection, and interplay. *Frontiers in Microbiology*, 7:422.

Piñeiro, A. and Barja, I. (2012). The plant physical features selected by wildcats as signal posts: an economic approach to fecal marking. *Naturwissenschaften*, 99:801–809.

Putman, R. J. (1984). Facts from faeces. *Mammal Review*, 14(2):79–97.

Rifkin, J. L., Nunn, C. L., , and Garamszegi, L. Z. (2012). Do animals living in larger groups experience greater parasitism? a meta-analysis. *The American Naturalist*, 180(1):70–82.

Robertson, S. J., Goethel, A., Girardin, S. E., , and Philpott, D. J. (2018). Innate immune influences on the gut microbiome: lessons from mouse models. *Trends in Immunology*, 39(12):993–1004.

Rodgers, T. W. and Janečka, J. E. (2013). Applications and techniques for non-invasive faecal genetics research in felid conservation. *European Journal of Wildlife Research*, 59:1–16.

Rook, G. A. W. (2010). 99th dahlem conference on infection, inflammation and chronic inflammatory disorders: Darwinian medicine and the 'hygiene' or 'old friends' hypothesis. *Clinical and Experimental Immunology*, 160(1):70–79.

Rook, G. A. W., Raison, C. L., and Lowry, C. A. (2014). Microbial 'old friends', immunoregulation and socioeconomic status. *Clinical and Experimental Immunology*, 177(1):1–12.

Roose-Amsaleg, C., Brygoo, Y., and Harry, M. (2004). Ascomycete diversity in soil-feeding termite nests and soils from a tropical rainforest. *Environmental Microbiology*, 6(5):462–469.

Roper, T. J., Conradt, L., Butler, J., Christian, S. E., Ostler, J., and Schmid, T. K. (1993). Territorial marking with faeces in badgers (*Meles meles*): A comparison of boundary and hinterland latrine use. *Behaviour*, 127(3/4):289–307.

Rose, C., Parker, A., and B. Jefferson, a. E. C. (2015). The characterization of feces and urine: a review of the literature to inform advanced treatment technology. *Critical Review in Environmental Science and Technology*, 45(17):1827–1879.

Rosengaus, R. B., Mead, K., Comb, W. S. D., Benson, R. W., and Godoy, V. G. (2013). Nest sanitation through defecation: antifungal properties of wood cockroach feces. *Naturwissenschaften*, 100:1051–1059.

Rowland, J. T. (1975). Some ecological aspects of animal excrement. *The American Biology Teacher*, 37(3):154–157.

Ruibal, M., Peakall, R., and Claridge, A. (2010). Socio-seasonal changes in scent-marking habits in the carnivorous marsupial *Dasyurus maculatus* at communal latrines. *Australian Journal of Zoology*, 58(5):317–322.

Sakamaki, T. (2010). Coprophagy in wild bonobos (*Pan paniscus*) at Wamba in the Democratic Republic of the Congo: a possibly adaptive strategy? *Primates*, 51:87–90.

Sato, Y., Saito, Y., and Sakagami, T. (2003). Rules for nest sanitation in a social spider mite, *Schizotetranychus miscanthi* Saito (Acari: Tetranychidae). *Ethology*, 109(9):713–724.

Scott, M. D. and Cordaro, J. G. (1987). Behavioral observations of the dwarf sperm whale, *Kogia simus*. *Marine Mammal Science*, 3(4):353–354.

Soave, O. and Brand, C. D. (1991). Coprophagy in animals: a review. *The Cornell Veterinarian*, 1(4):357–364.

Sujada, N., Sungthong, R., and Lumyong, S. (2014). Termite nests as an abundant source of cultivable actinobacteria for biotechnological purposes. *Microbes and Environments*, 29(2):211–219.

Tobey, J. R., Andrus, C. H., Doyle, L., Thompson, V. D., and Bercovitch, F. B. (2006). Maternal effort and joey growth in koalas (*Phascolarctos cinereus*). *Journal of Zoology*, 268(4):423–431.

Traniello, J. F., Rosengaus, R. B., and Savoie, K. (2002). The development of immunity in a social insect: evidence for the group facilitation of disease resistance. *Proceedings of the National Academy of Sciences*, 99(10):6838–6842.

Tso, I.-M. (1998). Stabilimentum-decorated webs spun by *Cyclosa conica* (Araneae, Araneidae) trapped more insects than undecorated webs. *The Journal of Arachnology*, 26(1):101–105.

Vencl, F. V., Gómez, N. E., Ploss, K., and Boland, W. (2009). The chlorophyll catabolite, pheophorbide a, confers predation resistance in a larval tortoise beetle shield defense. *Journal of Chemical Ecology*, 35:281–288.

Vilane, B. (2010). Assessment of stabilisation of adobes by confined compression tests. *Biosystems Engineering*, 106(4):551–558.

Wada-Katsumata, A., Zurekc, L., Nalyanya, G., Roelofs, W. L., Zhangf, A., and Schal, C. (2015). Gut bacteria mediate aggregation in the german cockroach. *Proceedings of the National Academy of Sciences*, 112(21):15678–15683.

Wald, C. (2016). The secret history of ancient toilets. *Nature*, 533(7604):456–458.

Walsh, P. T., McCreless, E., and Pedersen, A. B. (2013). Faecal avoidance and selective foraging: do wild mice have the luxury to avoid faeces? *Animal Behaviour*, 86(3):559–566.

Waltner-Toews, D. (2013). *The origin of feces: what excrement tells us about evolution, ecology, and a sustainable society.* ECW Press, Toronto.

Wang, J.-W., Kuo, C.-H., Wang, F.-C. K. Y.-K., Hsu, W.-H., Yu, F.-J., Hu, H.-M., Hsu, P.-I., Wang, J.-Y., and Wu, D.-C. (2019). Fecal microbiota transplantation: Review and update. *Journal of the Formosan Medical Association*, 118:S23–S31.

Wargo, J. A. (2020). Modulating gut microbes. *Science*, 369(6509):1302–1303.

Weiss, M. R. (2006). Defecation behavior and ecology of insects. *Annual Reviews of Entomology*, 51:635–661.

Wood, T. (1988). Termites and the soil environment. *Biology and fertility of soils*, 6(3):228–236.

Xu, Z., Liu, Z., Dong, X., Hu, T., Wang, L., Li, J., Liu, X., and Sun, J. (2018). Fecal microbiota transplantation from healthy donors reduced alcohol-induced anxiety and depression in an animal model of chronic alcohol exposure. *Chinese Journal of Physiology*, 61(6):360–371.

Yamamoto, I. (1984). Latrine utilization and feces recognition in the raccoon dog, *Nyctereutes procyonoides*. *Journal of Ethology*, 2:47–54.

Zala, S. M., Potts, W. K., and Penn, D. J. (2004). Scent-marking displays provide honest signals of health and infection. *Behavioral Ecology*, 15(2):338–344.

Zoberi, M. H. and Grace, J. K. (1990). Fungi associated with subterranean termite *Reticulitermes flavipes* in Ontario. *Mycologia*, 82(3):289–294.

Zub, K., Theuerkauf, J., Jedrzejewski, W., Jedrzejewski, B., Schmidt, K., and Kowalczyk, R. (2003). Wolf pack territory marking in the Bialowieza Primeval Forest (Poland). *Behaviour*, 140:635–648.

Zyskowski, K. and Prum, R. O. (1999). Phylogenetic analysis of the nest architecture of neotropical ovenbirds (Furnariidae). *The Auk*, 116(4):891–911.

3

Fecal mass control pathogens but don't enhance disease resistance in mound termites

3.1 Abstract

Some termite species have a fecal compartment present in their nests. This compartment is known to mediate extended disease resistance for such termites, because it harbours beneficial microbes which impair the growth of pathogens, preventing infection. However, this hypothesis was tested in a termite species from the older Higher Termites group, that construct their nest with feces and wood. The mechanisms behind this antimicrobial action, however, are still under scrutiny. Pathogens could be impaired (i) by competitive exclusion from beneficial microorganisms, or (ii) by direct supression originated from substances present in the the fecal mass, be those liberated from microorganisms or from termite metabolism itself. Here we verified these two hypotheses, using the fecal mass of *C. cumulans*' nests to evaluate its effect on the survival of these termites exposed to pathogens. We also used this fecal mass with microbes present and suppressed to investigate whether these microbes affect the germination of the termitopathogen. We found that the fecal mass (i) promotes an increment in the survival of termites, (ii) does not prevent the pathogenic infection in the body of termites, but (iii) it decreases the pathogen's germination. This decreasing effect is not only due to the presence of fecal mass' microbes, but also to the some yet unknown component of the fecal mass. These results demonstrate that the fecal mass of some termites does not act improve individual immunity, but it can contribute to their overall health. Moreover, it can impair the development of microbes in the nest, even if the beneficial microorganisms therein resident would be elliminated.

Keywords: Infection. Pathogenic microbes. Prophylaxis. Social insects. Soil.

3.2 Introduction

A vast diversity of animals, plants, and microbes are often found forming symbiotic link with termites (Roose-Amsaleg et al., 2004; Costa et al., 2009; Joseph et al., 2013). Such a link is particularly tight for the case of microbes inhabiting the termite gut (Brune and Dietrich, 2015). As with the termite body holding symbionts in an specialized compartment – the gut –, some termite nests have a distinct "waste" compartment rich in organic matter, soil and termite feces were microbes pullulate (Chouvenc et al., 2013; Costa et al., 2013; Barbosa-Silva et al., 2016; Moreira et al., 2018).

While exerting their action in cellulose degradation (Brune, 2014), termites gut microbes also excrete chemical compounds that control pathogens and increase the host' disease resistance (Rosengaus et al., 2011; Sujada et al., 2014; Peterson and Scharf, 2016). Likewise, microbes inhabiting the fecal nest's compartment of *C. formosanus* (Isoptera: Rhinotermitidae) provide disease resistance to their cohabiting termite individuals (Chouvenc et al., 2013). This has led Chouvenc et al. (2013) to postulate the "extended disease resistance hypotheses", which holds that the fecal nest compartment in *Coptotermes spp.* would have a function of increasing the disease resistance of individuals in addition to its sanitary role as a waste dump.

The mechanisms behind this antimicrobial action, however, are still under scrutiny, focusing on a species from the Higher Termites group, *C. cumulans* (Isoptera: Termitidae). Such termites belong to one of the most recent branches in the phylogeny of Isoptera, the Syntermitinae. Despite having large nests as *C. formosanus*, unlike these latter, *C. cumulans* mixes feces with soil –rather than wood– to build the internal nest compartment. In doing so, *C. cumulans* would be more exposed to entomopathogenic soil as bacterias, nematodes, and fungi from the genera *Beauveria*, *Isaria* (Cordycipitaceae) and *Metarhizium* (Clavicipitaceae) (Bruck, 2009; Vega et al., 2009; Peng et al., 2019).Puzzling enough, when opening a *C. cumulans* nest in the field, we always observe most of the juvenile individuals gathered at this fecal compartment (Grassé, 1958), as if it was a "nursery". All these inherent traits of *C. cumulans* biology seem to oppose a healthy lifestyle.

While asking why *C. cumulans* would keep such a potential source of infections inside their nest, considering their biology, we hypothesize that the nests' fecal mass would (i) make termites "stronger" so that they resist better to infections, or (ii) it make the pathogen "weaker", retarding its growth and, hence, preventing nest infections. Pathogens could be impaired by competitive exclusion from beneficial microorganisms, or by direct supression originated from substances present in the the fecal mass, be those exsudates from microorganisms or from termite metabolism itself. Here we verified these two hypotheses, that is to say, here we tested two distinct sets of mechanisms, one focused on the increment of individual immunity by the feces stored in the nest and another focused on the prophylactic role from this specialized fecal nest compartment. To do so, we inspected the effect of the nest fecal mass on the survival of *C. cumulans* individuals exposed to *Metarhizium anisopliae* and also the effects of the fecal mass, with and without active microoganisms, on the *in-vitro* growth of this fungus.

3.3 Material and Methods

3.3.1 Termite focal species and natural history

The *C. cumulans* (Kollar, 1832) is one of the mandibulate nasute termites that compose the newest group of termites, the so-called Higher Termites (Emerson, 1952; Constantino, 2002). *C. cumulans* feed on ground grass litter and mainly soil substrate (Donovan et al., 2001). The colony of *C. cumulans* is composed of two castes, the reproductive caste, with one queen and one king, and the non–reproductive caste, with thousands of soldiers and workers (Grassé, 1958). The reproductive caste keeps the production of new individuals and the non–reproductive caste act on the maintenance of the colony and the nest (Roisin, 2000). While termite soldiers and workers are normally seen in all nest galleries, it is in the galleries composing the fecal compartment that youngsters thrive (Grassé, 1958).

The nest of *C. cumulans* occurs on the soil surface in open grassland areas in Brazil, Bolivia, Paraguay, and Argentina (Redford, 1984; Constantino, 2002; Cosarinsky, 2011). The nest is ovoid (Grassé, 1958; Cosarinsky, 2011), whose bottom stays implanted underground while most of its body protrudes above-ground. Its above-ground height can reach just above two meters in height, one meter in diameter at the ground base. Nests attaining a few more than 400 liters in volume are commonly found in the field (J. A. Roxinol, personal observation, November 25, 2019).

This nest structure is bipartite, with a core and an external part (Grassé, 1958; Cosarinsky, 2011). The core, hereafter called fecal mass, is a compartment rich in soil, grass litter, termite feces, and saliva (Menezes et al., 2017). This part has larger galleries, similar to a carton hive (Grassé, 1958; Cosarinsky, 2011), where the juvenile termites (nymphs) thrive along with abundant and diverse microbial community (Grassé, 1958; Costa et al., 2013; Menezes et al., 2017). Such microbes are present in less amount in the soil adjacent to the nest and in the termite gut (Costa et al., 2013). The outward nest part forms a dry and cemented soil wall with threadlike and irregular galleries, that act as a hard outer shell to the colony (Cosarinsky, 2011). This outward wall can be as thick as five to 25 cm thickness in direct proportion to the nest volume and colony ontogeny (Guimarães, 2018).

3.3.2 Termites and nest fecal mass sampling

The nest fecal mass, worker and soldier termites were sampled from 18 undamaged nests found between 10 to 1000 meters apart at the municipality of Viçosa, Minas Gerais state, Brazil (Table 3.2). No pest management procedures have been recorded in the sampling area, no inquiline termite species were found within these nests. Termite individuals presented no noticeable damage or behavioral change before the trial started. Trials started later in the same day of the sampling. Survival trials used samples from 4 nests and were carried out in December 2019. The *in vitro* trials used samples from 15 nests (one of them previously sampled for survival trials – Table 3.2), and were carried out in August 2020.

The cold chisel and the gardening trowel tools used to open the nest, were disinfected with 70% alcohol and flamed immediately before each nest sampling. All nest were opened digging in their wall a hole $\varnothing \leq 30$ cm, at about 30% of the nest height from the ground and oriented to the east position. The fecal mass was sampled using a disinfected forceps and stored in sterilized falcon tubes immediately after the nests were opened. Termites present in fecal mass were removed before the fecal mass storage. After fecal mass storage, termites were also sampled and stored in boxes with fragments of the nest (using a distinct box for each nest) and were immediately taken to the laboratory for screening. In the laboratory, the termites from each box were collected with a forceps and placed inside an individual Petri dish with a lid. Then, they were transferred to the Petri dishes with the respective treatment settings.

3.3.3 Fungal spore preparation

The *M. anisopliae* was selected as a model organism for the trials based on their potential pathogenicity to termites, and because it occurs naturally in the soil and sometimes in termite nests (Kramm et al., 1982; Milner et al., 1998;

Myles, 2002; Neves and Alves, 2004; Zimmermann, 2007). This *M. anisopliae* strain is pathogenic and lethal to termites and other insects, but it is not a commercial strain.

Aiming to certify that the *M. anisopliae* spores emerged from a termite host, freeze-dried isolates of this *M. anisopliae* strain were previously cultivated in a *C. cumulans* carcass. The body surface of this carcass was previously subjected to antisepsis, to avoid other fungi contaminations. This antisepsis was carried out by submerging the carcass individually in a solution of 6% of hypochlorite sodium and sterilized distilled water. After being handily shaken for 10 seconds, the carcass were washed twice in sterilized distilled water, by submersion in microtubes. Then, these carcasss were placed in a Petri dish layered with PDA (Potato Dextrose Agar – KASVI, Madrid, ES – 39 gr of PDA in one liter of distilled water – concentration used herein all trials) with 0.002% of Chloramphenicol 25.0 mg. After the *M. anisopliae* germination over the PDA medium and carcass, a sample of this fungus was taken and seeded again in another Petri dish layered with PDA and Chloramphenicol. Lastly, we took isolates from this Petri dish to prepare a pure fungal stock suspension and use it subsequently according to each trial setup.

The stock suspension was prepared with 0.01 g of *M. anisopliae* spores in 20 ml of sterilized distilled water and 0.1% Tween 80 surfactant. Spores counting was ran adding 10 μl suspension in a hemocytometer at 400× of magnification. The *M. anisopliae* conidia concentration from the stock suspension used herein all trials was 2.5×10^8 conidia/ml. All these preparation of the fungal stock suspension were done according to Rosengaus and Traniello (1997).

3.3.4 Termite survival trial

Would the termites' nest fecal mass make termites stronger so that they resist to pathogen infections? To address this question, we exposed termites to *M. anisopliae* and inspected their survival in the presence and absence of their nest fecal mass. We also performed trial in the absence of pathogens and nest fecal mass to assess the termites' baseline survival.

Survival trials were performed on groups composed of one soldier plus 15 workers. Each group was sampled from a single termite colony. A total of four nests were used (Table 3.2). Termites were confined in Petri dish plates. This plates had each one of the two half parts with a idividual factor under test. These halves part were previuosly layered with a fungal PDA growth medium. Termites moved freely above the halves – factors – inside Petri dishes. Treatments consisted of (i) *M. anisopliae* seeded over PDA in one half of the plate, with fecal mass over PDA on the other half; (ii) *M. anisopliae* seeded over PDA in one half of the plate, with the other half free of fecal mass – just PDA; (iii) fecal mass over PDA on the one half, with the other half free of *M. anisopliae* – just PDA ; and (iv) the control – no *M. anisopliae*, no fecal mass, just PDA in the halves (Figure 3.1). The plates had $\varnothing = 60$ mm, height = 15 mm, and the termites' density inside the plate was kept near 0.14 (that is, 14% of the plate's surface was occupied by the bodies of the termites themselves, leaving 86% of the space free), aiming to avoid density bias effect on termites behavior (Miramontes and DeSouza, 2008).

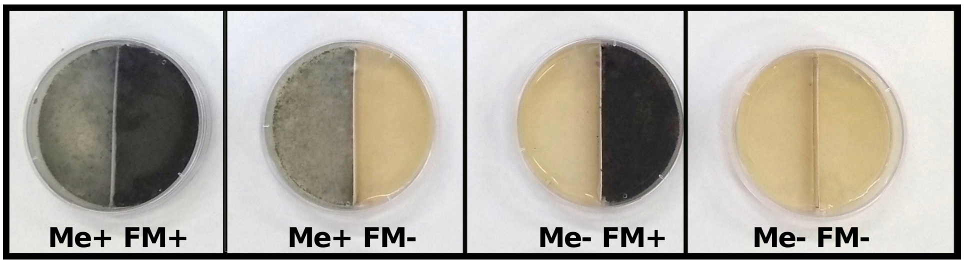

Figura 3.1: Experimental setup where termite groups were subjected to the survival trial. Treatments were arranged combining the presence (+) or absence (-) of *Metharizium anisopliae* (Me) and termite nest fecal mass (FM) on each half of the plate. In the absence of one of these factors, just sterilized PDA growth medium was used.

Each half of the plate received 1.5 ml of solidified and sterilized PDA medium with 0.002% of Chloramphenicol 25.0 mg. *M. anisopliae* fungus was previously cultivated until sporulation (five days). This cultivation was done inoculating 10 μl of the stock suspension of *M. anisopliae* directly in one half side of the plate and pouring 1.5 ml of sterilized PDA medium above it. Fecal mass treatments were prepared applying 1 g of grounded nest fecal mass over the Petri dish half containing sterilized and solidified PDA medium. The nest fecal mass was added to the plate immediately before the beginning of the survival trial. The fecal mass used in each plate came from the same nest as the termites under test. All preparations were done inside a flow chamber using sterilized objects so that to minimize external contamination.

Survival observations were carried out every one hour, counting the number and noting the caste of dead individuals inside each plate until all individuals were dead. Survival evaluation began 30 minutes after termites were placed in the Petri dishes with the treatments in a BOD incubator, at 25 °C ±1 °C in complete darkness. Three replicates for each treatment and each nest have been used.

The *causa mortis* of individuals was inspected following the Rosengaus and Traniello (1997). To do so, dead individuals were placed over a plate previously layered with PDA medium so that microbes thereby grown could be identified. The first, the seventh, and the fourteenth individual found dead in each plate had its *causa mortis* determined.

The body surface of termites carcass was subjected to antisepsis before *causa mortis* plating. This antisepsis was carried out as done before in the *C. cumulans* carcase to fungal stock suspension preparation (Vide section 3.3.2). After antisepsis, the carcases were plated in the center of the plate on a 3 ml layer of sterilizedd and solidified PDA medium with 0.002% of Chloramphenicol 25.0 mg. A sterilized cotton ball ($\cong$ 0.1 g) soaked on 1 ml of distilled water was placed at the internal edge of the plate to increase the moisture and favor the fungi growth. The plates ($\varnothing = 30$ mm, height = 15 mm) were sealed by wrapping them in cling film after plating the carcases. These plates were incubated at 25 °C ±1 °C in complete darkness until the microbes grew and covered the termite carcass (five to 10 days after plating).

The *causa mortis* could then be identified. *M. anisopliae* infection was identified by comparison to the fungal growth pattern in control plates containing previously aseptic termites carcases which were submerged in *M. anisopliae* suspension (Supplementary material – Figure 3.5). *Causa mortis* could then be classified as "*M. anisopliae*" or "other".

3.3.5 *In vitro* trial

Here, we addressed the following questions: Would the nest fecal mass make the termite pathogens weaker by affecting their growth? If it does, would this weakening occur due to the presence of antagonic microbes in the fecal mass or due to some intrinsic physical and chemical trait of the nest fecal mass itself? To answer these questions, we carried out two *in vitro* trials comparing the growth of termite pathogen fungus (*M. anisopliae*) in the presence of (i) sterilized, (ii) non sterilized nest fecal mass with and and (iii) in the absence of it. This was done using the method bellow as adapted from Rosengaus et al. (1998, 2000) and Cole et al. (2020).

We prepared individual suspensions from each sample of nest fecal mass, adding one gram of grounded nest fecal mass in 20 ml of sterilised 0.1% TWeen 80 surfactant. A second suspension from the same sample was prepared using fecal mass which was sterilised by microwaves irradiation. Microwaves irradiation were used as an alternative to sterilising methods with chemicals or high temperature, because it cause less alteration in chemical composition of substances (Darbar and Lakzian, 2007; Shareef et al., 2019). The effectivity of such a sterilizing method has been shown by Islam and Weil (1998). To do so, one gram of grounded nest fecal mass was placed in a glass plate and irradiated in a 1400 W household-type microwave oven, with max energy power at 2450 Hz in the continuous mode for two minutes. These irradiated nest fecal mass were so added to the 20 ml of TWeen 80 surfactant in sterilized falcon tubes.

Suspensions were so handly shook for one minute to foster detachment of putative microbes and other possible compounds from the nest fecal mass. Subsequently, 60 μl of supernatant suspension of either irradiated or non-irradiated fecal mass was incubated in microtubes with 60 μl of the *M. anisopliae* stock suspension for 24 hours at 25 °C on a rotating plate (150 rpm) in a BOD incubator and under darkness. A mix of 60 μl *M. anisopliae* suspension and 60 μl TWeen 80 was used as the control treatment to estimate the baseline of the *M. anisopliae* conidia germination.

After incubation, 10 μl of each suspension was spread onto a microscope slide containing 1 ml of sterilized and solidified PDA growth medium. Each sample was run in triplicate, three slides per nest, and treatment. Another replicate slide was prepared and inspected immediately, aiming to determine if *M. anisopliae* conidia had germinated during the 24 hours incubation period. The seeded slides were cultured for 18 hours at 25 °C ±1 °C inside glass plates lined with one sheet of towel paper moistured with 3 ml of distilled water to keep high moisture content. The microscopic slides were placed on top of overturned plates and inside another glass plate avoiding direct contact with the moistened paper sheets. These glasses plates were also sealed wrapping on it with cling film to avoid external contamination. Immediately after 18 hours, conidia germination was evaluated counting the proportion of germinated *M. anisopliae* conidia in 10 focal fields per slide, at 400× magnification (Supplementary material – Figure 3.4).

3.3.6 Statistical analysis

The analysis of the survival trial inspected whether treatments (*M. anisopliae* + fecal mass, *M. anisopliae* alone, control) would affect the meantime elapsed from the experiment onset until a termite worker was found dead in the plate. The data were subjected to censored survival analysis under the Weibull distribution (Martinussen and Scheike, 2006), using the *"survival"* package in R software (version 3.4.4). Modeling proceeded by building a full model, including the three treatment setups, plus the nest identity as a blocking term.

Model simplification by backward stepwise deletion was carried out aiming to inspect consequent changes in deviance (Crawley, 2015). After only significant terms persisted in the model, further simplification was done by amalgamating levels within categorical variables one by one from the full model in the crescent order of the meantime to death.

For the *in vitro* trial, we contrasted the average of germination rate *M. anisopliae* in the presence of three treatments: non-irradiated fecal mass; irradiated fecal mass; and control. Germination rate were evaluated 24 hours after the beginning of incubations in liquid suspensions and 18 hours after seeding on slides. The average rate of conidia germination of each treatment was obtained from three replicate slides per nest. The germination rate in each slide was obtained from ten random focal fields. Analyses were carried out under Generalized Linear Modeling using R software (version 3.4.4).

3.4 Results

3.4.1 Termite survival trial

Infection by *M. anisopliae* was confirmed as the *causa mortis* of termites in treatments containing this pathogen (Supplementary material – Table 3.1). Termites exposed to *M. anisopliae* died faster than those not exposed to it (the control treatment), and this deleterious effect of the fungus was not affected by the presence of fecal mass (Figure 3.2 and Supplementary material – Table 3.4). During the evaluation of this trials, we also observed that some termite workers bury carcases with fecal mass (Figure 3.6). In addition, the treatment type and nest identity significantly affected the termites' survival (Supplementary material – Table 3.3), and the fecal mass positivelly affected termites' survival (Figure 3.2). Statistical differences between treatment levels are given in Supplementary material – Table 3.4.

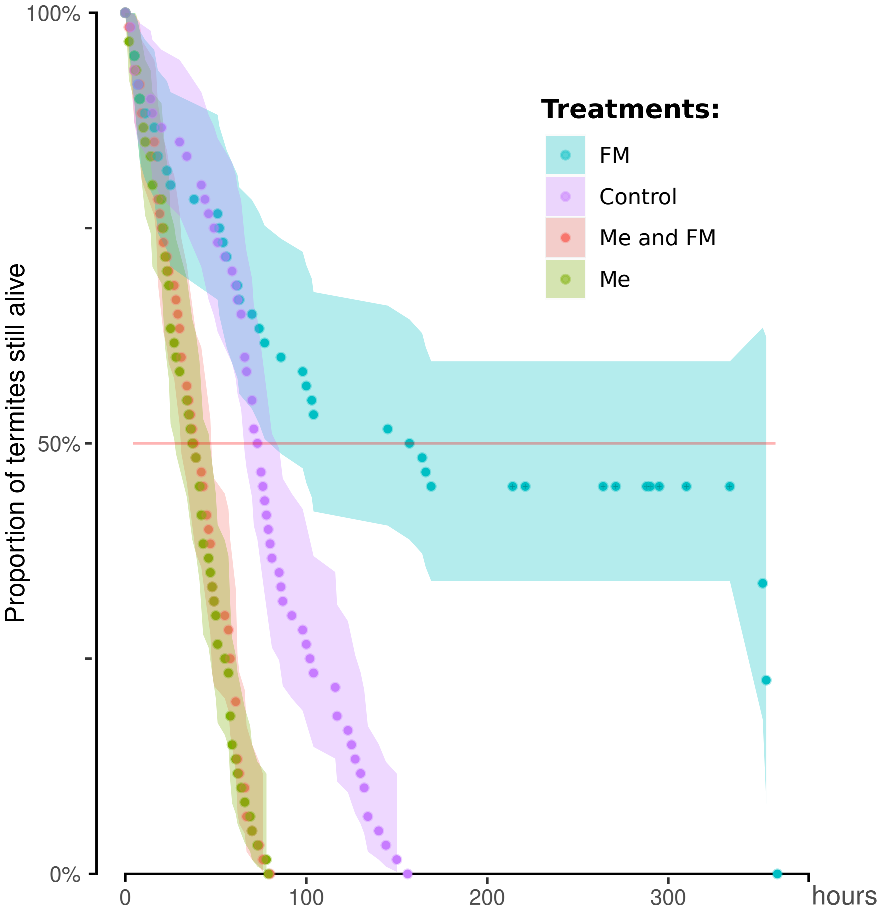

Figura 3.2: Workers of C. cumulans termites exposed to *M. anisopliae* died faster than those not exposed to it (p<.001). Nest fecal mass did not protect termites from mortality by *M. anisopliae* infection. Axes depict the proportion of termites still alive in each treatment (x-var) as time elapses since the begining of the experiment (x-var). Dots are the readings performed at each occasion and colored clouds indicate the confidence intervals (95%) for each curve). FM = just Fecal Mass exposition; Me = *M. anisopliae* exposition; Me + FM = *M. anisopliae* plus nest Fecal Mass exposition; and the Control = absence both *M. anisopliae* and nest Fecal Mass.

3.4.2 *In vitro* trial

The germination of *M. anisopliae* was negatively affected by the presence of fecal mass ($p = 0.0003$) after 42 hours of interaction. Interesting, non-irradiated fecal mass was more deleterious to *M. anisopliae* than was irradiated fecal mass ($p = 4.069 \times 10^{-10}$), indicating the existence of at least two factors of fungal deterrence: one that is sensitive to irradiation (resident microbes, for instance) and another insensitive to it (Figure 3.3). *M. anisopliae* conidia do not germinated before 24 hours of incubation in all treatment setups.

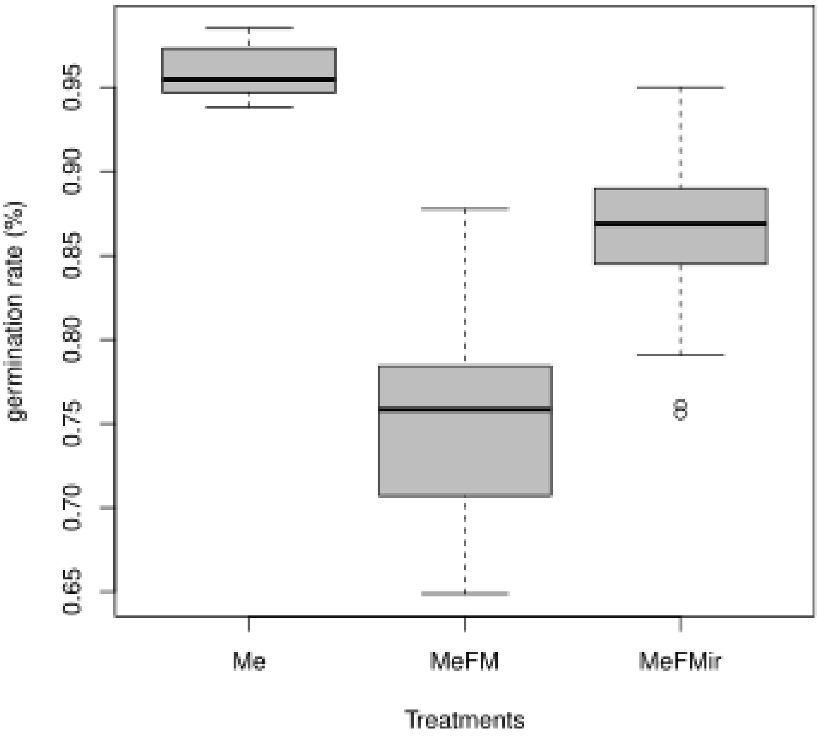

Figura 3.3: Termite nest fecal mass decreased the germination rate of *M. anisopliae* conidia F(3, 30) = 65.76, p = 1.08^{-11}. The germination was lower when the microbes were present in the fecal mass (that is, when the fecal mass was not irradiated) F(2, 31) = 82.54, p = 4.069^{-10}. Dots are averages of 3 readings, each one being, itself, an average of 10 focal fields in a given slide. Data from 15 nests. Me = suspension of *M. anisopliae* alone, Me + FM = suspension of *M. anisopliae* incubated with non-irradiated fecal mass, Me + FMir = suspension of *M. anisopliae* incubated with irradiated fecal mass

3.5 Discussion

Our results pointed to a neutral effect of the termite nest fecal mass in strengthening termite individuals against pathogen infection and mycosis (Figure 3.2), which contradicted our first hypothesis. However, the results supported the second hypothesis, since the termite nest fecal mass decreased the germination of the termitopathogen fungus (Figure 3.3). We also found that the microbes naturally residing in the fecal mass were able to deter the germination of *M. anisopliae*, because non-irradiated fecal mass – with higher microbes content – was more effective in impairing *M. anisopliae* than irradiated one (Figure 3.3). Furthermore, because *M. anisopliae* deterrence still persisted in irradiated fecal mass, it seems appropriate to state the fecal mass can control pathogens even after the resident beneficial microbiota was elliminated.

These results suggest that if *M. anisopliae* conidia are taken from outside to the core of the nest, their germination will be hindered by the fecal mass microbes and by some other unknown factor associated to the fecal mass. Whether or not these factors would be exsudates from a previous population of beneficial microbes or even from termite metabolism itself, its beyond the scope of this work. In the end, keeping feces inside the nest enhances colony success in impairing epizootic risks in the nest. However, the fecal mass could not increase the resistance of termites to disease when these individuals were already stricken by the pathogenic infection (Figure 3.3). So, the nest fecal mass can be said to have a prophylactic role that is exerted at the level of the super-organism – the colony – but not directly in the individuals, as previously asserted by Chouvenc et al. (2013). Once individuals are infected, the fecal mass can not impair infections to build up.

It is also interesting to note that, even when microbes are elliminated (by irradiation, Fig. 3.3), the prophylactic action of the fecal mass persist, even though at a lower extent. This seem to indicate that the protection offered by the fecal mass seem not to rely entiretly on the actual presence of live colonies of beneficial microbes. It is tempting to hypothesise on the origin of this prophylaxis: on one hand, it might be simple accumulation of exudates from the beneficial microbes themselves. On the other hand, one could suspect that antimicrobial metabolites from termite origin could also be carried over along with excretes. Clearly, these are hypotheses still in need of test. We provide them here not only show the complexity of the mechanisms behind Chouvenc's extended disease resistance, but also point out the breadth of research avenues opened by these results.

Additionally, the negative effect of the nest fecal mass and the microbes on the germination of termitopathogen may also explain why *C. cumulans* youngsters are so numerous in the fecal mass nest compartment (Grassé, 1958): since their immune system is still under development (Traniello et al., 2002), it would be safer for them to gather in a nest compartment where pathogen pullulation is suppressed. If this suppression takes the pathogenic load to a nonlethal dose, the fecal mass can be acting as the immune priming mechanism that increase youngsters resistance to pathogens (Sadd and Schmid-Hempel, 2006; Gálvez and Chapuisat, 2014; Sheehan et al., 2020). In this particular sense, the fecal mass compartment is comparable to a clean nursery were youngsters are protected against infections.

We also observed during the data sampling of the survival trial some *C. cumulans* workers burying some carcases with the fecal mass (Figure 3.6). This observation highlight that the termites can use the fecal mass as an ally to fight the spread of pathogens. In fact, many termites species bury carcases to minimize odor interference in their chemical communication (Chouvenc et al., 2012), and also to control pathogen pullulation (Rosengaus et al., 1998).

On a more ecological tone, this negative effect of the fecal mass and their microbes on the pathogen germination seems potentially relevant to the success of the Termitidae family, the more speciose and abundant termite group

(Bucek et al., 2019; Engel, 2019). Maybe not coincidentally, this special nest compartment is present in Termitidae species usually holding large populations, such as *Macrotermes bellicosus* and our focus here, *C. cumulans* (Engel, 2019; Mizumoto and Bourguignon, 2020). This notion seems to find support on the fact that, among Lower Termites, those having enormous success also have such intimate contact with their own feces: *C. formosanus* is very abundant and widespread (Redford, 1984; Cosarinsky, 2011).

It is also tempting to admit a link between the negative effect of the fecal mass and their microbes on the germination of pathogens from the soil – here observed – and the prevalence of fecal mass in soil-made nests, as the *C. cumulans* case. Such a link may be particularly tighter for the case of Higher Termites that are more prone to pathogens threats by living and feed in the soil. After all, if the pathogens are suppressed their harm in the termite colony is decreased, so more individuals would survive, and larger populations be maintained. New research may disentangle the identity of the agents enrolled within this symbiosis and the evolutionary implications of this relationship on these species.

In conclusion, rather than being a waste structure, the fecal mass compartment in *C. cumulans* nests seem to play a role in decreasing the reproductive efficiency of soil termitopathogens. Such a role is played mainly due to the presence of microbes in this mass. Here, we argue that in the Higher Termites the fecal mass seems more effective in playing a prophylactic role against pathogens would found in the nest structure itself than in enhancing metabolic resistance of individuals to disease. That is, the fecal mass and their microbes can control soil pathogen carried to the nest core, but they do not enhance disease resistance direction onto the metabolism of termites individuals.

3.6 Bibliography

Barbosa-Silva, A. M., Farias, M. A. A., de Mello, A. P., de Souza, A. E. F., Garcia, H. H. M., and Bezerra-Gusmão, M. A. (2016). Lignocellulosic fungi in nests and food content of *Constrictotermes cyphergaster* and *Inquilinitermes fur* (Isoptera, Termitidae) from the semiarid region of Brazil. *Fungal Ecology*, 20:75–78.

Brauman, A., Bignell, D. E., and Tayasu, I. (2000). *Termites: evolution, sociality, symbioses, ecology.*, chapter Soil-feeding termites: biology, microbial associations and digestive mechanism, pages 233–259. Springer, Dordrecht.

Bruck, D. J. (2009). The ecology of fungal entomopathogens. *BioControl*, 55:103–112.

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews Microbiology*, 12(3):168–180.

Brune, A. and Dietrich, C. (2015). The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annual Review of Microbiology*, 69(1):145–166.

Bucek, A., Šobotník, J., He, S., Shi, M., McMahon, D. P., Holmes, E. C., Roisin, Y., Lo, N., and Bourguignon, T. (2019). Evolution of termite symbiosis informed by transcriptome-based phylogenies. *Current Biology*, 29(21):3728–3734.

Chouvenc, T., Efstathion, C. A., Elliott, M. L., and Su, N.-Y. (2013). Extended disease resistance emerging from the faecal nest of a subterranean termite. *Proceedings of the Royal Society B: Biological Sciences*, 280(1770):1– 9.

Chouvenc, T., Robert, A., Sémon, E., and Bordereau, C. (2012). Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. *Insectes Sociaux*, 59:119–125.

Cole, E. L., Bayne, H., and Rosengaus, R. B. (2020). Young but not defenseless: Antifungal activity during embryonic development of a social insect. *Royal Society Open Science*, 7(8):191418.

Constantino, R. (2002). The pest termites of South America: taxonomy, distribution and status. *Journal of Applied Entomology*, 126(7-8):355–365.

Cosarinsky, M. I. (2011). The nest growth of the neotropical mound-building termite, *Cornitermes cumulans*: A micromorphological analysis. *Journal of Insect Science*, 11(1):122.

Costa, D. A., de Carvalho, R. A., de Lima Filho, G. F., and Brandão, D. (2009). Inquilines and invertebrate fauna associated with termite nests of *Cornitermes cumulans* (Isoptera, Termitidae) in the Emas National Park, Mineiros, Goiás, Brazil. *Sociobiology*, 53(2):443–453.

Costa, P. S., Oliveira, P. L., Chartone-Souza, E., and Nascimento, A. M. A. (2013). Phylogenetic diversity of prokaryotes associated with the mandibulate nasute termite *Cornitermes cumulans* and its mound. *Biology and Fertility of Soils*, 49:567–574.

Crawley, M. J. (2015). *Statistics: an introdution using R*. John Wiley & Sons, secound edition.

Darbar, S. R. and Lakzian, A. (2007). Evaluation of chemical and biological consequences of soil sterilization methods. *Caspian Journal of Environmental Sciences*, 5(2):87–91.

Donovan, S. E., Eggleton, P., and Bignell, D. E. (2001). Gut content analysis and a new feeding group classification of termites. *Ecological Entomology*, 26(4):356–366.

Emerson, A. E. (1952). The neotropical genera *Procornitermes* and *Cornitermes* (Isoptera, Termitidae). *Bulletin of the American Museum of Natural History*, 99(8):475–540.

Engel, M. S. (2019). Termite evolution: a primal knock on wood or a hearty mouthful of dirt. *Current Biology*, 29(21):R1126–R1129.

Grassé, P.-P. (1958). Sur le nid et la biologie de *Cornitermes cumulans* (Kollar), termites brésilién. *Insectes Sociaux*, 5(2):189–199.

Guimarães, M. P. M. (2018). A manutenção da muralha: o investimento diferencial no reparo de danos em paredes de cupinzeiros. Master's thesis, Universidade Federal de Viçosa, Viçosa, Brazil.

Gálvez, D. and Chapuisat, M. (2014). Immune priming and pathogen resistance in ant queens. *Ecology and Evolution*, 4(10):1761–1767.

He, S., Sieksmeyer, T., Che, Y., Mora, M. A. E., Stiblik, P., anjd Mark C. Harrison, R. B., Šobotník, J., Wang, Z., Johnston, P. R., and McMahon, D. P. (2020). Evidence for reduced immune gene diversity and activity during the evolution of termites. *bioRxiv.* doi: 10.1101/2020.07.09.192013.

Islam, K. and Weil, R. (1998). Microwave irradiation of soil for routine measurement of microbial biomass carbon. *Biology and Fertility of Soils*, 27(4):408–416.

Ji, R., Kappler, A., and Brune, A. (2000). Transformation and mineralization of synthetic 14c-labeled humic model compounds by soil-feeding termites. *Soil Biology and Biochemistry*, 32(8-9):1281–1291.

Joseph, G. S., Seymour, C. L., Cumming, G. S., Cumming, D. H., and Mahlangu, Z. (2013). Termite mounds as islands: woody plant assemblages relative to termitarium size and soil properties. *Journal of Vegetation Science*, 24(4):702–711.

Kramm, K. R., West, D. F., and Rockenbach, P. G. (1982). Termite pathogens: Transfer of the entomopathogen *Metarhizium anisopliae* between *Reticulitermes sp.* termites. *Journal of Invertebrate Pathology*, 40(1):1–6.

Martinussen, T. and Scheike, T. H. (2006). *Dynamic regression models for survival data.* Statistics for Biology and Health. Springer Science + Business Media, Inc., New York, 1 edition.

Menezes, L., Alvarez, T. M., Persinoti, G. F., Franco, J. P., Squina, F., Moreira, E. A., Paixão, D. A. A., Costa-Leonardo, A. M., da Silva, V. X., Clerici, M. T. P. S., et al. (2017). Food storage by the savanna termite *Cornitermes cumulans* (Syntermitinae): A strategy to improve hemicellulose digestibility? *Microbial Ecology*, 76(2):492–505.

Milner, R. J., Staples, J. A., Hartley, T. R., Lutton, G. G., Driver, F., and Watson, J. A. L. (1998). Occurrence of *Metarhizium anisopliae* in nests and feeding sites of australian termites. *Mycological Research*, 102(2):216–220.

Miramontes, O. and DeSouza, O. (2008). Individual basis for collective behaviour in the termite, *Cornitermes cumulans*. *Journal of Insect Science*, 8(1):1–22.

Mizumoto, N. and Bourguignon, T. (2020). Modern termites inherited the potential of collective construction from their common ancestor. *Ecology and Evolution*, 10(13):6775 – 6784.

Moreira, E. A., Alvarez, T. M., Persinoti, G. F., Paixão, D. A. A., Menezes, L. R., Cairo, J. P. F., Squina, F. M., Costa-Leonardo, A. M., Carrijo, T., and Arab, A. (2018). Microbial communities of the gut and nest of the humus- and litter-feeding termite *Procornitermes araujoi* (Syntermitinae). *Current Microbiology*, 75:1609–1618.

Myles, T. (2002). Isolation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) from *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) with convenient methods for its culture and collection of conidia. *Sociobiology*, 40(2):257 – 264.

Neves, P. M. O. J. and Alves, S. B. (2004). External events related to the infection process of *Cornitermes cumulans* (kollar) (Isoptera: Termitidae) by the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae. Neotropical Entomology*, 33(1):51–56.

Peng, T. L., Syazwan, S. A., and Lee, S. H. (2019). *Microbes for sustainable insect pest management - an eco-friendly approach*, volume 1 of *Sustainability in plant and crop protection*, chapter Soil-borne entomopathogenic bacteria and fungi, pages 23–41. Springer.

Peterson, B. F. and Scharf, M. E. (2016). Lower termite associations with microbes: synergy, protection, and interplay. *Frontiers in Microbiology*, 7:422.

Redford, K. H. (1984). The termitaria of *Cornitermes cumulans* (Isoptera, Termitidae) and their role in determining a potential keystone species. *Biotropica*, 16(2):112–119.

Roisin, Y. (2000). Diversity and evolution of caste patterns. In *Termites: evolution, sociality, symbioses, ecology*, pages 95–119. Springer.

Roose-Amsaleg, C., Brygoo, Y., and Harry, M. (2004). Ascomycete diversity in soil-feeding termite nests and soils from a tropical rainforest. *Environmental Microbiology*, 6(5):462–469.

Rosengaus, R. B., A., J. F. T., Lefebvre, M. L., and Carlock, D. M. (2000). The social transmission of disease between adult male and female reproductives of the dampwood termite *Zootermopsis angusticollis. Ethology, Ecology and Evolution*, 12(4):419 – 433.

Rosengaus, R. B., Guldin, M. R., and Traniello, J. F. A. (1998). Inhibitory effect of termite fecal pellets on fungal spore germination. *Journal of Chemical Ecology*, 24:1697–1706.

Rosengaus, R. B. and Traniello, J. F. A. (1997). Pathobiology and disease transmission in dampwood termites *Zootermopsis angusticollis* (Isoptera: Termopsidae)] infected with the fungus *Metarhizium anisopliae* (Deuteromycotina: Hypomycetes). *Sociobiology*, 30(2):185–195.

Rosengaus, R. B., Zecher, C. N., Schultheis, K. F., Brucker, R. M., and Bordenstein, S. R. (2011). Disruption of the termite gut microbiota and its prolonged consequences for fitness. *Applied and Environmental Microbiology*, 77(13):4303–4312.

Sadd, B. M. and Schmid-Hempel, P. (2006). Insect immunity shows specificity in protection upon secondary pathogen exposure. *Current Biology*, 16(12):1206–1210.

Shareef, S. A., Hamasaeed, P. A., and Ismaeil, A. S. (2019). Sterilization of culture media for microorganisms using a microwave oven instead of autoclave. *Rafidain Journal of Science*, 28(1):1–6.

Sheehan, G., Farrell, G., and Kavanagh, K. (2020). Immune priming: the secret weapon of the insect world. *Virulence*, 11(1):238–246.

Sujada, N., Sungthong, R., and Lumyong, S. (2014). Termite nests as an abundant source of cultivable actinobacteria for biotechnological purposes. *Microbes and Environments*, 29(2):211–219.

Traniello, J. F., Rosengaus, R. B., and Savoie, K. (2002). The development of immunity in a social insect: evidence for the group facilitation of disease resistance. *Proceedings of the National Academy of Sciences*, 99(10):6838–6842.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., Koike, M., Maniania, N. K., Monzón, A., Ownley, B. H., Pell, J. K., Rangel, D. E. N., and Roy, H. E. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecology*, 2(4):149–159.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology*, 17(9):879–920.

3.6 Supplementary Material

Tabela 3.2: Characteristics of the nests where the termites and the fecal mass were sampled

Identity	Geodesic coordinates	Altitude (m)	Height (cm)	Volume (L)[a]	Trial
Nest 01	20°46’59.05”S 42°50’42.79”W	702	86	168.2	survival
Nest 02	20°46’59.20”S 42°50’42.72”W	705	83	243.1	survival
Nest 03	20°46’57.20”S 42°50’47.00”W	706	51	108.8	survival
Nest 04	20°47’00.30”S 42°50’42.90”W	698	71	147.8	surv. & germ.
Nest 05	20°46’57.10”S 42°50’47.10”W	706	75	212.9	germination
Nest 06	20°47’00.50”S 42°50’54.80”W	764	97	412.8	germination
Nest 07	20°46’58.70”S 42°50’49.00”W	716	88	248.2	germination
Nest 08	20°46’58.20”S 42°50’51.10”W	737	71	117.2	germination
Nest 09	20°46’58.40”S 42°50’55.60”W	745	81	167.3	germination
Nest 10	20°47’00.60”S 42°50’53.10”W	758	91	130.6	germination
Nest 11	20°47’01.10”S 42°50’53.30”W	764	102	319.5	germination
Nest 12	20°46’59.70”S 42°50’47.00”W	728	107	335.0	germination
Nest 13	20°46’59.60”S 42°50’48.40”W	712	80	228.2	germination
Nest 14	20°46’54.90”S 42°50’23.60”W	705	62	191.2	germination
Nest 15	20°46’53.00”S 42°50’22.60”W	737	121	142.5	germination
Nest 16	20°46’54.40”S 42°50’21.20”W	731	106	471.0	germination
Nest 17	20°46’55.60”S 42°50’20.60”W	732	107	451.5	germination
Nest 18	20°46’57.70”S 42°50’20.20”W	727	51	102.0	germination

[a] The nest volume was calculated according to (Guimarães, 2018).

Tabela 3.1: Results from the *Causa mortis* trial

Nest Identity	**Treatment**	**Death Sequence**	***Causa mortis***
Nest 01	Control	First	Another
Nest 02	Control	First	Another
Nest 03	Control	First	Another
Nest 04	Control	First	Another
Nest 01	Control	Seventh	Another
Nest 02	Control	Seventh	Another
Nest 03	Control	Seventh	Another
Nest 04	Control	Seventh	Another
Nest 01	Control	Fourteenth	Another
Nest 02	Control	Fourteenth	Another
Nest 03	Control	Fourteenth	Another
Nest 04	Control	Fourteenth	Another
Nest 01	MeBM	First	Another
Nest 02	MeBM	First	Another
Nest 03	MeBM	First	Another
Nest 04	MeBM	First	*M. anisopliae*
Nest 01	MeBM	Seventh	Another
Nest 02	MeBM	Seventh	Another
Nest 03	MeBM	Seventh	*M. anisopliae*
Nest 04	MeBM	Seventh	*M. anisopliae*
Nest 01	MeBM	Fourteenth	*M. anisopliae*
Nest 02	MeBM	Fourteenth	Another
Nest 03	MeBM	Fourteenth	*M. anisopliae*
Nest 04	MeBM	Fourteenth	*M. anisopliae*
Nest 01	Me	First	*M. anisopliae*
Nest 02	Me	First	Another
Nest 03	Me	First	*M. anisopliae*
Nest 04	Me	First	Another
Nest 01	Me	Seventh	*M. anisopliae*
Nest 02	Me	Seventh	*M. anisopliae*
Nest 03	Me	Seventh	*M. anisopliae*
Nest 04	Me	Seventh	*M. anisopliae*
Nest 01	Me	Fourteenth	*M. anisopliae*
Nest 02	Me	Fourteenth	*M. anisopliae*
Nest 03	Me	Fourteenth	*M. anisopliae*
Nest 04	Me	Fourteenth	*M. anisopliae*

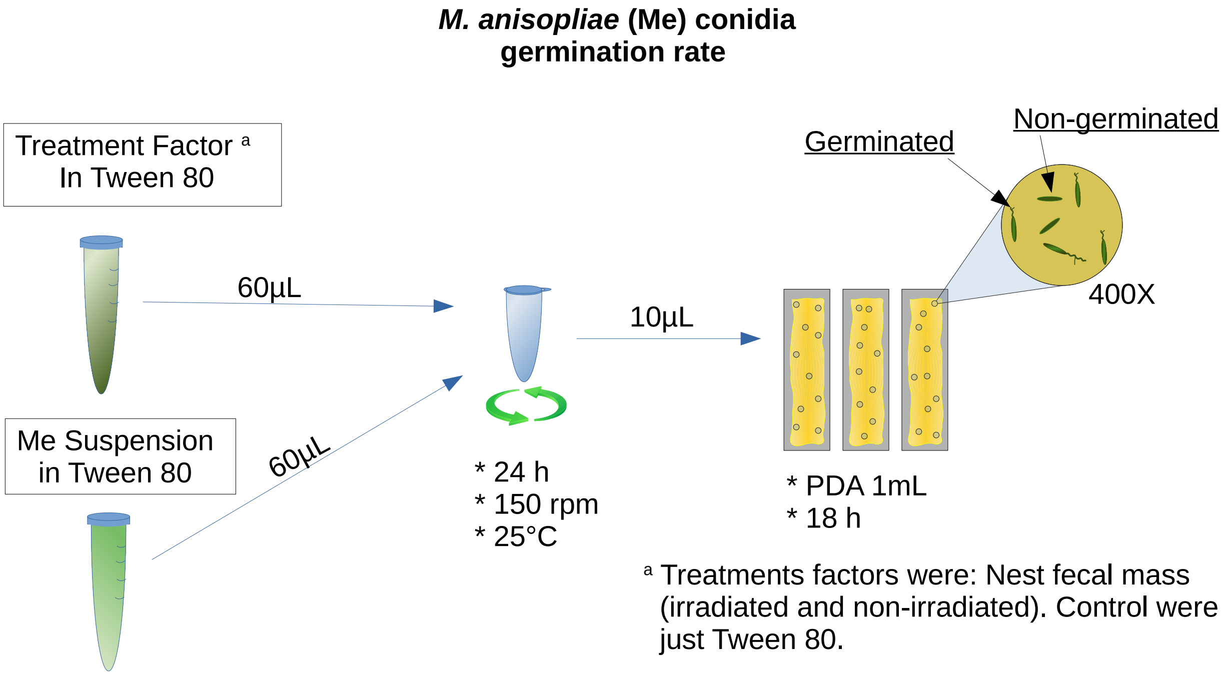

Figura 3.4: Experimental setup to quantify *M. anisopliae* conidia germination rate, run in the termite nest fecal mass and termitopathogen fungus interaction trial.

Tabela 3.3: Analysis of deviance results from the effect of nest fecal mass on *C. cumulans* survival exposed to the pathogen *M. anisopliae*. See Material & Methods for more details.

	Df	**Deviance**	**Resid. Df**	**-2∗LL**[a]	**Pr ($>\chi^2$)**
Null	NA	NA	238	2376.75	NA
Treatments	3	167.73	235	2209.02	3.93^{-36}
Nest identity	3	70.35	232	2138.67	3.60^{-15}

[a] LL = log–likelihood.

Tabela 3.4: Results of analysis of deviance from the model simplification by stepwise deletion from the effects of nest fecal mass on *C. cumulans* survival exposed to the pathogen *M. anisopliae*. See Material & Methods for more detail.

Model[a]	**Terms**[b]	**Resid. Df**	**-2∗LL**[c]	**Test Df**	**Deviance**	**Pr ($>\chi^2$)**
Model 1	All treatments	235	2209.02	−3	−70.36	3.60^{-15}
Model 2	Me+FM- and Me+FM+	236	2209.134	−1	−0.12	0.73
Model 3	Me+FM+ = Control	237	2245.68	−1	−36.54	1.49^{-9}
Model 4	Control = Me-FM+	237	2268.96	−1	−59.82	1.04^{-14}

[a] Model built in the model simplification by stepwise deletion.

[b] Terms combined in the model. Terms abreviation meanings are: Me + FM = presence of *M. anisopliae* and nest fecal mass in the same treatment; Me = Just the presence of *M. anisopliae* in the treatment.

[c] LL = log–likelihood.

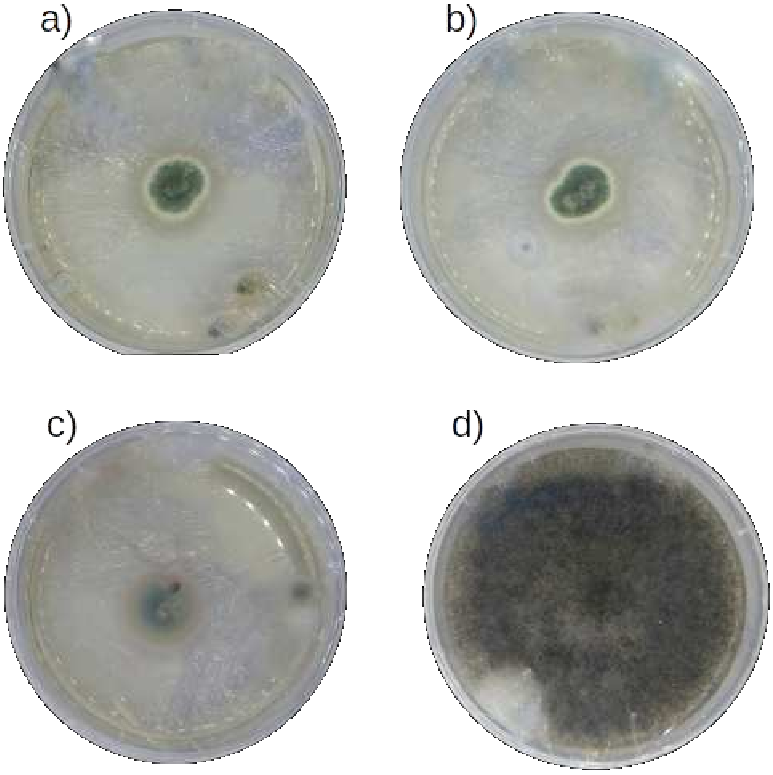

Figura 3.5: Causa Mortis paterns. a) Me infection, treatment Me alone; b) Me infection, control; c) and d) Another microbe infection, treatment Me and FM.

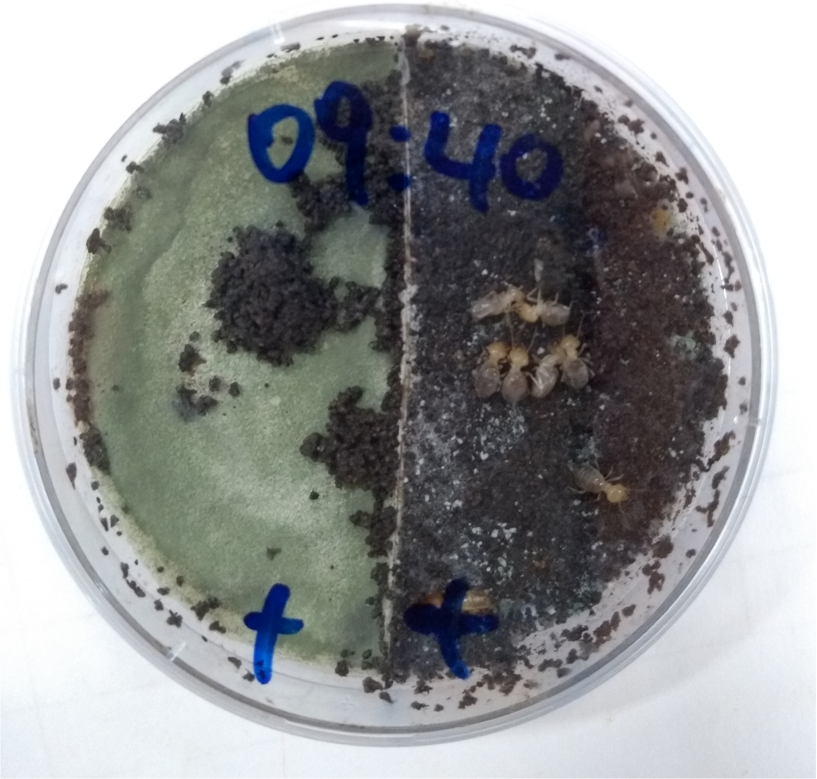

Figura 3.6: Termites carcase buried with fecal mass. Dark mass pile, more visible in left side.

4

Conclusões Gerais

A partir dos trabalhos apresentados nesta tese, concluímos que a importância das fezes para os animais vai muito além de ser a etapa final do processo digestivo. As fezes são utilizadas pelos animais como meio de comunicação, construção, sistema de defesa e até como alimento e estratégia profilática. Dentre os animais que mais utilizam as fezes de conspecíficos, os cupins são um grande destaque. Apresentamos aqui que um dos motivos que possibilitam o uso e contato direto dos cupins com suas fezes é devido ao papel das fezes na ação profilática contra a infecção de patógenos no ninho. Esse papel é desempenhado principalmente pelos micróbios residentes na massa fecal dos ninhos por diminuírem a capacidade reprodutiva de fungos termitopatógenos. Para o caso de *C. cumulans*, a massa fecal e seus micróbios atuam prevenindo a infecção de patógenos, entretanto ela não é capaz de aumentar a resistências dos indivíduos à doenças.