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Interactions between entomopathogenic fungi and arthropod hosts: Immune response of *Noctua pronuba* larvae and gut bacteriome dynamics of *Rhipicephalus microplus*

Thaís Almeida Corrêa

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FEDERAL RURAL UNIVERSITY OF RIO DE JANIERO
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Under the Supervision of Professor
Ph.D. Patrícia Silva Gôlo

And Co-supervision of Professors
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And ***Ph.D. Deborah Henderson***

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INTERAÇÕES ENTRE FUNGOS ENTOMOPATOGÊNICOS E HOSPEDEIROS ARTRÓPODES:RESPOSTA IMUNE DE LARVAS DE *Noctua pronuba* E DINÂMICA DO BACTERIOMA INTESTINAL DE *Rhipicephalus microplus*

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Sob a supervisão da Professora
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Tese submetida como requisito parcial para a obtenção do grau de **Doutora em Ciências no Programa de Pós-Graduação em Ciências Veterinárias.**

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"Have I not commanded you?
Be strong and courageous.
Do not be afraid; do not be discouraged, for the Lord your God will be with you wherever you go."
(Joshua 1:9)

"Believe in yourself, trust, and make it happen!"

(*Telma Dantas - Mother*)

I dedicate this work to my mother, Telma Dantas, and to my grandparents, Alfredo Almeida and Georgina Dantas (in memoriam), for their effort, dedication, and encouragement of my studies, which made it possible for me to achieve my dreams.".

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BIOGRAPHY

Thaís Almeida Corrêa, daughter of Telma Dantas dos Santos Almeida and granddaughter of Alfredo dos Santos Almeida and Georgina Dantas dos Santos Almeida, was born on November 15, 1991, in Rio de Janeiro, Brazil. She completed her high school education at São Sebastião School in Rio de Janeiro, graduating in 2008.

In 2013, she enrolled in Veterinary Medicine at the Federal Rural University of Rio de Janeiro (UFRRJ), completing her degree in December 2018. During her undergraduate studies, she was awarded a Scientific Initiation Scholarship (PIBIC-CNPq) from 2015 to 2018 at the Microbial Control Laboratory under the supervision of Professor Patrícia Silva Gôlo.

In March 2019, she began her Master's degree in the Graduate Program in Veterinary Sciences (PPGCV) at UFRRJ, continuing under the supervision by Dr. Patrícia. Following her academic formation, she started her PhD studies in March, 2021 supervised by Dr. Patrícia Silva Gôlo and co-supervised by Dr. Huarrisson Azevedo Santos and Dr. Deborah Henderson. During her Ph.D., September to December 2023, she worked as a substitute professor in the Department of Animal Production at UFRRJ and she conducted part of her research at Kwantlen Polytechnic University through the ELAP – Emerging Leaders in the Americas Program, under the supervision of Dr. Henderson in 2024. In addition, she received the "FAPERJ Nota 10" fellowship in 2025, a prestigious award granted to graduate students in recognition of outstanding academic excellence.

Throughout her academic career, she has actively participated in numerous national ("Congresso Brasileiro de Parasitologia Veterinária – CBPV" and "Simpósio de Controle Biológico" - SICONBIOL) and international (Society for Invertebrate Pathology - Annual Conference) congresses in her field of research, attended professional development courses, and published abstracts and scientific papers with her research group in specialized journals.

ABSTRACT

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The control of arthropod vectors and agricultural pests requires sustainable alternatives in light of the limitations and impacts associated with chemical acaricides and insecticides. In this context, entomopathogenic fungi are key elements both in shaping host immune responses and as part of integrated management strategies. This thesis investigated two distinct models: (i) the cattle tick *Rhipicephalus microplus*, focusing on the interaction of *Metarhizium anisopliae* with the gut bacteriota in the presence or absence of *Anaplasma marginale*, and (ii) the moth *Noctua pronuba*, an important agricultural pest, with emphasis on its humoral immune interactions with *Beauveria bassiana* and *Trichoderma atroviride*. In Chapter I, the gut bacterial diversity of engorged *R. microplus* females was characterized through a metabarcoding approach targeting the V3–V4 region of the bacterial 16S rRNA gene in groups exposed or not to *M. anisopliae* and infected with *A. marginale*. Coinfection reshaped the bacteriota, with a higher number of associated taxa and a distinct microbial composition compared to control and singly infected groups. Co-occurrence network analysis revealed that the coinfected microbiome (*Metarhizium* and *Anaplasma*) exhibited greater connectivity and robustness, suggesting a compensatory response of the host and bacterial community to dual stress rather than beneficial stability. Functional prediction indicated that these shifts may represent a metabolic adaptation of the bacterial consortium to support tick and/or pathogen survival, supported by the increased abundance of functional genes associated with metabolic pathways in the coinfected group. These findings highlight the modulatory role of the gut bacteriota at the fungus–vector–pathogen interface, with direct implications for the vector competence of *R. microplus*, and reinforce the potential of fungi to interfere with pathogen transmission cycles. In Chapter II, the immune response of *N. pronuba* larvae was investigated after exposure to *B. bassiana* (entomopathogenic) and *T. atroviride* (non-entomopathogenic). The differential expression of innate immunity-related genes (*Cecropin1*, *Attacin*, and *Peptidoglycan recognition protein A - PGRP-A*) was quantified by qPCR. *B. bassiana* induced a early activation of *Attacin* and *PGRP-A* six hours after treatment, whereas *T. atroviride* promoted suppression of *Attacin* and *PGRP-A* at 12 and 24 hours, suggesting an ability to interact with host immune pathways despite lacking evident pathogenicity. These results expand our understanding of recognition and defense mechanisms against fungi and underscore the importance of exploring novel fungal isolates, such as *Trichoderma*, in biocontrol programs. The two chapters demonstrate that arthropod immune responses and gut bacteriota dynamics are central determinants in the interaction with entomopathogenic fungi and pathogens. Understanding these relationships provides a foundation for developing innovative biocontrol strategies capable of reducing dependence on chemical inputs and contributing to sustainability in animal health and agriculture.

Keywords: *Rhipicephalus microplus*; *Noctua pronuba*; entomopathogenic fungi; *Trichoderma atroviride*; gut bacteriota; innate immunity; *Anaplasma marginale*; biocontrol

LIST OF TABLES

CHAPTER I

CHAPTER II

LIST OF FIGURES

LITERATURE REVIEW

CHAPTER I

SUMMARY

GENERAL INTRODUCTION

Arthropods of agricultural and veterinary relevance exert a profound economic and ecological impact worldwide, largely due to their role as crop pests or vectors of pathogens. Two representative species this dual importance: the cattle tick *Rhipicephalus microplus*, the most harmful ectoparasite affecting bovine production in tropical and subtropical regions, and the moth *Noctua pronuba*, an invasive lepidopteran pest causing significant crop losses in temperate regions (Barré; Uilenberg, 2010; DiFonzo; Russell, 2010). Despite belonging to distinct ecological niches, both species highlight the pressing need for sustainable management strategies, as reliance on chemical control has resulted in resistance, environmental contamination, and threats to non-target organisms (Manosathiyadevan *et al*., 2017; Klafke *et al*., 2024).

In this scenario, biological control using entomopathogenic fungi has gained prominence as an environmentally friendly alternative. *Metarhizium anisopliae* and *Beauveria bassiana* are the most studied and commercially applied fungi, recognized for their ability to infect and kill a wide variety of arthropods, including ticks and insects of agricultural concern (Fernandes; Bittencourt; Roberts, 2012; Mascarin; Jaronski, 2016). Their pathogenicity arises from multiple mechanisms, including cuticle penetration, toxin production, and modulation of host defenses (Ortiz-Urquiza; Keyhani, 2013). With green agricultural development, people are urgently seeking safe, effective, and environmentally friendly plant disease control measures. Biological control is mainly used to control harmful organisms in plants through beneficial organisms and their products to control plant diseases and effectively reduce the application of chemical fertilizers and pesticides (Harman *et al*., 2021). Alongside these classical entomopathogens, *Trichoderma atroviride*, traditionally employed as a biofungicide for its antagonistic effects against phytopathogenic fungi, has emerged as a versatile candidate (Poveda, 2021; Dwisandi *et al*., 2024).

The host response to fungal infection is central to the outcome of these interactions. In insects such as *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Chilo suppressalis* (Lepidoptera: Crambidae), the immune system relies on innate defenses, including recognition receptors and antimicrobial peptides, which are rapidly modulated upon exposure entomopathogenic fungi (Shamakhi *et al*., 2019; Wang *et al*., 2010). However, immune responses may differ depending on whether the challenge. In parallel, ticks such as *R. microplus* harbor a complex gut microbiota that interacts with pathogens, as *Anaplasma*, and influencing

immune homeostasis and vector competence (Narasimhan; Fikrig, 2015; O'Neal *et al*., 2021; de La Fuente *et al*., 2017; Pérez *et al*., 2025). Together, immunity and microbiota represent critical interfaces where fungi exert their modulatory effects, shaping host survival and pathogen transmission.

The first chapter focuses on *R. microplus*, evaluating how treatment with *M. anisopliae* affects the gut bacteriome under infection by *Anaplasma marginale*, the etiological agent of bovine anaplasmosis (Kocan *et al*., 2010; O'Neal *et al*., 2021). By analyzing bacterial community composition and predicted functions, this work provides insights into how fungal biocontrol may influence microbial networks and, consequently, tick physiology and vector capacity. The second chapter investigates *N. pronuba* larvae, characterizing their immune humoral response to *B. bassiana* and *T. atroviride*. Through gene expression analyses, it demonstrates the ability of the insect to discriminate between pathogenic and non-pathogenic fungi, as well as the capacity of both species to modulate humoral immune responses.

Taken together, these chapters converge on a common theme: entomopathogenic fungi not only infect arthropod hosts but also reshape their immune and bacterial 'landscapes". By addressing both microbiota dynamics in ticks and immune gene regulation in insects, this thesis emphasizes the versatility and applicability of fungal agents in biocontrol. The integration of these findings reinforces the potential of fungi such as *Metarhizium*, *Beauveria*, and *Trichoderma* as sustainable tools in pest and vector management. Beyond their direct pathogenic effects, their ability to modulate host defenses and microbial communities positions them as valuable components in the development of innovative, multifunctional bioproducts for agriculture and livestock.

LITERATURE REVIEW

1.1 Arthropods of interest in animal and agricultural health: biological aspects and economic impact

1.1.1 Biology and life cycle of *Rhipicephalus microplus*

Rhipicephalus microplus (Canestrini, 1887) is a tick belonging to the family Ixodidae, widely distributed across tropical and subtropical regions of the Americas, Africa, Asia, and Australia (Barré; Uilenberg, 2010), where it finds ideal conditions for its development, particularly due to the presence of its preferred host, cattle. This species, classified within the class Arachnida and subclass Acari, is an obligate ectoparasite with a monoxenous life cycle, meaning it completes all its parasitic stages on a single host (Monteiro, 2017).

The taxonomy of *R. microplus* has undergone significant revisions over time. Initially classified as *Boophilus microplus*, morphological and phylogenetic analyses revealed that the genus *Boophilus* was paraphyletic about *Rhipicephalus*, meaning it did not include all descendants of a common ancestor. Based on these findings, Murrell and Barker (2003) proposed synonymizing *Boophilus* with *Rhipicephalus*, retaining it as a subgenus. Consequently, the species was reclassified as *Rhipicephalus (Boophilus) microplus*, reflecting its phylogenetic position within the *Rhipicephalus* genus.

The life cycle of *R. microplus* comprises two main phases: a parasitic phase and a free-living phase. After hatching, larvae migrate to the top of vegetation searching for a host. This behavior is driven by their negative geotropism, which orients them vertically toward the tips of grasses and other forage plants. In this strategic position, they remain until they detect the approach of a host through chemical and physical cues perceived by specialized sensory structures, especially the Haller's organ located on the tarsi of the forelegs (Sonenshine; Roe, 2013). Upon encountering a host, they attach primarily to thin and vascularized skin areas, such as the ears, neck, and perineum. On the host, the larvae feed on blood and molt into nymphs, which also feed before molting into adults. After mating, females continue feeding until fully engorged. They then detach and fall to the ground, where oviposition occurs (Furlong; Prata, 2005) (Figure 1). The pre-oviposition period lasts from one to three days after detachment, followed by the deposition of thousands of eggs (which may exceed 3,000 per female). Egg incubation takes about 21 days, depending on temperature and humidity. The larvae emerge and wait for a new host, thus completing the cycle (Furlong; Prata, 2005; Monteiro, 2017). Under optimal conditions, the complete life cycle can last approximately 21 to 28 days.

This life cycle is highly sensitive to environmental factors and can be directly affected

by climate change, particularly global warming. As an ectothermic arthropod, *R. microplus* responds quickly to variations in temperature and humidity. Studies have shown that increasing temperatures can accelerate the development of both parasitic and non-parasitic stages, shorten the life cycle and increase the number of annual generations (Nuttall, 2022). Moreover, the rise in average temperatures and changes in precipitation patterns favor the expansion of the species' geographic distribution, enabling its colonization of previously unsuitable regions (Gray *et al*., 2009). This may intensify outbreaks of diseases such as bovine babesiosis and anaplasmosis and hinder the control of tick populations (Pérez-Otáñez *et al*., 2020). Therefore, understanding these interactions is essential to adapting parasite management strategies to the new climatic scenario.

Figure 1: Illustrative representation of the biological cycle of *Rhipicephalus microplus* in cattle. This tick is monoxenous, completing its parasitic stages, larva, nymph, and adult, on the same host. Once fully engorged, females detach from the animal to lay eggs in the environment (1). Larvae hatch from the eggs and ascend the vegetation in search of a new host, restarting the cycle (2). Larval development includes molting into the nymphal stage (3), which then transforms into male and female adults (4). Males seek out females for mating, which occurs while the latter are still feeding. As females feed intensively and reach full engorgement (5), they detach from the host and move to the ground to oviposit, thus restarting the species' life

cycle. Source: Original image by Lukevisuals/iStock. Adapted by Thaís Almeida Corrêa (2021).

1.1.2 Role of *Rhipicephalus microplus* as a pathogen vector

After mosquitoes, ticks - particularly those in the family Ixodidae - are considered the main vectors of diseases in humans, with notable public health relevance due to their ability to transmit various pathogens (de la Fuente *et al*., 2008; Sonenshine; Roe, 2013). They are also recognized as one of the most important vectors of pathogens affecting domestic and wild animals, especially in regions where they are endemic (Gray *et al*., 2009). *R. microplus* is widely recognized as one of the main vectors of hemoparasite pathogens affecting cattle, playing a critical role in the transmission of Bovine Parasitic Sadness (BPS). This disease complex includes the etiological agents *Babesia bovis*, *B. bigemina* (Almazán *et al*., 2022), and *Anaplasma marginale* (de la Fuente *et al*., 2008; Salinas-Estrella *et al*., 2022).

Narasimhan and Fikrig (2015) emphasize the role of ticks as important vectors, highlighting that their ecological and parasitic success is closely linked to their adaptation to a hematophagous life cycle and their ability to evade the host immune response. In this context, the salivary glands represent one of the main organs involved in the tick-pathogen interface. During feeding, tick attachment is accompanied by the secretion of saliva rich in immunomodulatory and anticoagulant molecules. This secretion not only modulates the host's immune response, facilitating feeding, but also enables pathogen transmission, promoting tick survival and perpetuating the life cycles of microorganisms. The salivary glands are not merely "channels" through which pathogens exit and enter the host. They actively respond to the presence of pathogens. In other words, the tick senses the infection and alters the cellular behavior of these glands. Studies have shown that this tissue not only facilitates the transmission of microorganisms but also responds directly to infection through changes in gene expression profiles (Šimo *et al*., 2017; Zivkovic *et al*., 2010). This means that salivary gland genes are differentially regulated (some are upregulated, others downregulated) when the tick is infected. For instance, in *R. microplus* infected with *A. marginale*, 279 differentially expressed genes were identified in the salivary glands (Zivkovic *et al*., 2010), demonstrating the complexity of the molecular adaptations involved and how the pathogen profoundly alters tick physiology, creating a more favorable environment for its survival and transmission.

From an evolutionary standpoint, pathogens such as *Anaplasma phagocytophilum* and *Borrelia burgdorferi* have developed sophisticated mechanisms to survive and replicate in tick

tissues by overcoming immune barriers. For example, *A. phagocytophilum* suppresses apoptosis in the salivary gland cells of its vector by modulating porins, thereby prolonging cell viability and enhancing its propagation (Ayllón *et al*., 2013). Moreover, this pathogen reorganizes the host cell cytoskeleton, facilitating its intracellular persistence and immune evasion (Abdullah *et al*., 2016). *B. burgdorferi*, in turn, exhibits resistance to hemocyte activity in *Ixodes scapularis*, bypassing phagocytosis and cell lysis responses (Dai *et al*., 2010). These examples illustrate how tick-borne pathogens act as coevolutionary agents that shape, and are shaped by, the physiological environment of the vector. Vector competence, therefore, is not merely a matter of pathogen presence but rather the result of a dynamic interaction between molecular, adaptive, and immune mechanisms that ensure the maintenance of the infectious cycle. Due to its central role in this interface, the salivary gland represents not only a key link in pathogen transmission but also a promising target for vector-blocking strategies (Villar *et al*., 2016; de la Fuente *et al*., 2017).

In addition to salivary glands, other tissues also play important roles in tick vector competence, such as the midgut. *R. microplus* harbors a complex gut microbiota, whose composition can directly influence its ability to acquire, maintain, and transmit pathogens (Castro *et al*., 2019; Bonnet *et al*., 2017). The digestive tract acts not only as an immune barrier but also as a site for microbial colonization and replication, as demonstrated in the present work, where *A. marginale* was detected in the midgut of *R. microplus* through qPCR. This finding reinforces the idea that vector competence involves multiple tissues and biological interactions. In this context, the gut microbiota also acts as a key component, capable of modulating the interaction between vector and pathogen (Zolnik *et al*., 2016; Narasimhan; Fikrig, 2015). Symbiotic bacteria present in ticks can influence the success of pathogenic colonization, either promoting or inhibiting this process (Bonnet *et al*., 2017; Bonnet *et al*., 2021). This complex tripartite relationship tick, microbiota, and pathogen, is an important research area for the development of biological control strategies and disease transmission prevention.

1.1.3 Impacts and control strategies for *Rhipicephalus microplus* in livestock production

In April 2025, Brazil remains the world's largest exporter of beef and the second-largest global producer, reaffirming its significance in the international livestock industry (USDA, 2025). According to the most recent data from the Brazilian Institute of Geography and Statistics (IBGE), the national cattle herd reached a record high of approximately 239 million

head in 2023. In comparison, when Grisi *et al*. (2014) estimated annual economic losses of USD 3.24 billion due to *R. microplus* parasitism, the herd consisted of 212.3 million animals. Considering the growth of the cattle population over the past decade and the continued reliance on conventional tick control practices with low technological renewal, it is reasonable to infer that current economic losses exceed those estimated in 2014. This underscores the urgent need for more effective and sustainable management strategies.

The biology of *R. microplus* directly impacts both beef and milk production (Marques *et al*., 2020). Its continuous blood feeding and prolonged attachment to the host can cause anemia, stress, and increase susceptibility to secondary infections (Rodriguez-Vivas *et al*., 2018). Moreover, engorgement and oviposition of females are key to the persistence of infestations in pastures, emphasizing the importance of interrupting the parasitic life cycle through effective control measures.

The sanitary importance of this tick is further highlighted by its role as a vector of hemoparasites that cause Bovine Babesiosis and Anaplasmosis, collectively known in Brazil as Bovine Parasitic Sadness (BPS), a major cause of cattle mortality (Furlong; Prata, 2005).

Historically, *R. microplus* control in Brazil has relied on the use of chemical acaricides. Currently, more than 250 commercial products are registered, including organophosphates, amidines, pyrethroids, phenyl pyrazoles, macrocyclic lactones, and isoxazolines (Klafke *et al*., 2024). However, intensive and often improper use of these compounds has led to widespread resistance among tick populations (Obaid *et al*., 2023), a phenomenon reported in several regions of the country (Higa *et al*., 2015; Klafke *et al*., 2017). The constant presence of *R. microplus* may also pose trade barriers, particularly due to the detection of acaricide residues in meat and milk, which can compromise the acceptance of Brazilian products in international markets with stringent sanitary standards. Resistance to six out of the seven acaricide classes currently available on the Brazilian market has been reported, especially in the states of Mato Grosso do Sul, Minas Gerais, and Rio Grande do Sul (Klafke *et al*., 2024). Factors contributing to resistance include repeated use of the same active ingredient, underdosing, application failures, lack of prior efficacy diagnostics on farms (Rodriguez-Vivas *et al*., 2018), or even the heritable reduction in sensitivity of tick populations to active substances, leading to decreased treatment efficacy over time (Devaney, 2013). According to the 2022 COMAC Yearbook (SINDAN), antiparasitic account for approximately 62% of the Brazilian veterinary pharmaceutical market across all animal species. Within this segment, products targeting ectoparasites in cattle, such as *R. microplus*, represent a significant share due to the parasite's

economic impact on national livestock production (EMBRAPA, 2021; SINDAN, 2022).

There is growing concern over the environmental impact of acaricides, particularly regarding contamination of natural resources and the presence of residues in animal products. Data from the Ministry of Agriculture show that acaricide residues are among the main contaminants detected in beef and dairy products (MAPA, 2023), highlighting the urgent need for safer and more sustainable control methods that meet the requirements of international consumer markets. One promising alternative is immunological control, which involves vaccinating the host with antigens capable of inducing a protective response and reducing tick infestations. This approach presents several advantages, including a favorable cost-benefit ratio, absence of withdrawal periods, and greater safety for both animals and consumers (Canales *et al*., 2009). Vaccines based on the Bm86 antigen have shown variable efficacy, and new targets, such as subolesin, are currently under investigation (Parizi *et al*., 2012; Arocho Rosario *et al*., 2022). Despite existing challenges, studies indicate that vaccination, when combined with rational use of acaricides, can enhance infestation control and reduce selective pressure for resistance (Parizi *et al*., 2012; Arocho Rosario *et al*., 2022).

In the absence of a national tick control program (Klafke *et al*., 2024), Integrated Parasite Management (IPM) has emerged as a promising approach, combining chemical, biological, and cultural measures. Strategies include rotating active ingredients, proper pasture management, selection of genetically resistant animals, and the use of biological control agents such as the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Camargo *et al*., 2016; Marciano *et al*., 2021). *R. microplus* control must be treated as a strategic issue for animal health, the competitiveness of the national livestock industry, and global food security. Advancing innovative, science-based, and farmer-adapted solutions is essential to address this challenge responsibly and effectively.

1.1.4 Biology and life cycle *Noctua pronuba*

The order Lepidoptera, encompassing both butterflies and moths, represents a highly diverse group of insects, with around 160,000 species described worldwide (Brady *et al*., 2021). Members of this order are closely linked to flowering plants and contribute significantly to ecosystem functions, including pollination, herbivory through leaf consumption, and seed dispersal (Hammond; Miller, 1998; Cook *et al*., 2022). *N. pronuba* (Linnaeus, 1758), commonly known as the large yellow underwing, is a Lepidopteran species belonging to the family Noctuidae, one of the most diverse and economically significant groups of herbivorous

insects (Mitchell *et al*., 2023). Originally native to Europe and Asia, the species was accidentally introduced into North America in the late 20th century and has since established itself as a major agricultural pest across much of the United States, especially in temperate regions such as southern Canada (Floate, 2017).

Adults have forewings with variable coloration, usually in shades of brown with small prominent black spots near the apex, while the hindwings are a striking orange-yellow. The wingspan ranges from 50 to 55 mm, which distinguishes them from other species. Larvae reach up to 40 mm in length, with a typically olive-brown body, occasionally greenish or with a reddish hue. Each body segment displays a distinctive black and cream-colored stripe on both sides of the dorsal midline. The brown-colored head features two thick black lines forming a “V” extending from the "neck" of the larva. These robust larvae can reach up to 50 mm in their final instar. They feed during mild winter days (temperatures around 7°C or higher), hence the common names “winter cutworm” or “snow cutworm” (Floate, 2017).

The life cycle of *N. pronuba* is holometabolous, consisting of egg, larva, pupa, and adult stages (Figueiredo *et al*., 2023). Females lay clusters of 200–300 eggs on host plants or nearby soil, which hatch in about 10 days under temperatures ranging from 4°C to 25°C (IAF-BC, 2024). The caterpillars feed on crops such as cereals (wheat, barley), vegetables (lettuce, cabbage), and pasture grasses. They are particularly damaging due to their habit of severing young plants at the base (Mitchell *et al*., 2023). The larval stage lasts from 4 to 8 weeks, undergoing up to six instars, followed by pupation in the soil, which can last 2–4 weeks (Eichlin; Cunningham, 2011). Unlike *N. comes*, whose larvae overwinter only during winter months, *N. pronuba* exhibits phenological plasticity: under cold conditions (<10°C), larval development can be extended up to 230 days before pupating underground (Wallis; Sisterson, 2024).

Adults require 4 to 6 weeks to mature before laying egg clusters and remain active until early October (Bechinski *et al*., 2009) (Figure 2). Typically, the species completes one generation per year, although three to four generations may occur in warmer climates (Passoa; Hollingsworth, 1996). Adults are attracted to artificial lights and are known for their strong dispersal capacity, migrating long distances in search of resources (Figueiredo *et al*., 2023). Data from Anderson (2024), collected in Vaccinium agroecosystems in British Columbia, showed that *N. pronuba* was the third most abundant species in light traps, with 46 individuals collected, 39 of which (85%) carried pollen grains, including Vaccinium pollen in 13 specimens. This pollen-carrying capacity, along with nocturnal flower visitation, suggests a dual ecological role: as a larval foliar pest and a potential adult pollinator (Ribas-Marques *et al*., 2022).

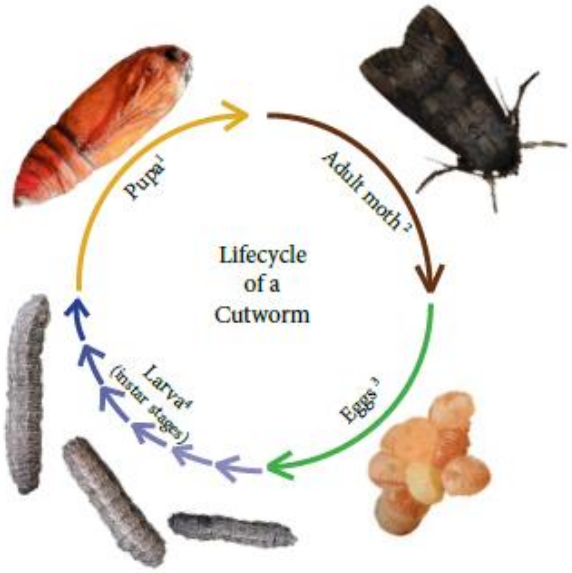

Figure 2: Cutworm lifecycle: illustrating its main stages: egg, larva (with multiple instars), pupa, and adult (moth). Arrows indicate the sequential transition between developmental stages. This species belongs to the order Lepidoptera and is considered a significant agricultural pest in various regions of the world. Source: USGS Bee Monitoring Lab; Reago, A.; McClarren, C.; Gavloski, J. – Manitoba Agriculture.

1.1.5 Economic and sanitary importance of *Noctua pronuba*

The agricultural relevance of *N. pronuba* lies in its capacity to cause significant economic losses, especially in cereal cropping systems (such as wheat, barley, oats, and rye), vegetables (including broccoli, cauliflower, carrot, onion, potato, spinach, sugar beet, and

tomato), and forage crops (such as alfalfa and grass hay), as well as other crops like strawberry, rhubarb, grape, chrysanthemum, and marigold in temperate regions of Europe and North America (Bechinski *et al*., 2009; Gómez *et al*., 2022). It is estimated that this species can cause losses ranging from 20% to 30% under severe infestations, with damage associated with the cutting of seedlings at or below the cotyledon level, as well as underground damage to root crops, where larvae feed on crowns and roots. At night, larvae climb the canopy of some host plants to feed on leaves, shoots, and flowers, while during the day they hide under crop residues or soil clods (Jacques *et al*., 2021).

Larvae exhibit either climbing or subterranean feeding behavior, which directly influences their detection and control. Management costs in British Columbia, Canada, can reach USD 12 to 15 per hectare per year. Due to the scarcity of specific studies on this species, comparisons with other Noctuidae species with similar habits, such as *Agrotis ipsilon* (Bourner; Cory, 2004) and *Spodoptera frugiperda* (Feistler *et al*., 2022), are often used to infer its impact and management strategies.

The management of *N. pronuba* is particularly challenging due to its subterranean feeding activity, which limits the efficacy of conventional foliar insecticides (Floate, 2017; Cutworm-booklet, 2017). Integrated strategies include monitoring with light traps for adults and soil sampling to detect larvae, allowing for targeted interventions (Jacques *et al*., 2021). Pest control is hindered by the larvae's subterranean behavior and increasing resistance to conventional insecticides, such as pyrethroids. Recent studies have identified a 40% reduction in sensitivity to lambda-cyhalothrin, requiring the development of more sustainable alternatives (Gómez *et al*., 2022). Among these alternatives, the use of biological control agents such as the entomopathogenic nematode *Steinernema carpocapsae* stands out, with proven efficacy against other subterranean Noctuidae species, such as *A. ipsilon*, achieving up to 72% larval mortality in moist soils without significant environmental impact (Gómez *et al*., 2022). Additionally, cultural practices such as crop rotation and proper soil preparation contribute to reducing larval populations in affected agricultural areas (Floate, 2017).

1.2 Entomopathogenic fungi as biological control tools in arthropods

1.2.1 History of biological control using entomopathogenic fungi

Biological control is an ancient practice involving using natural enemies to reduce populations of pests and vectors. Historically, this practice dates back to the 3rd century BC, when predatory ants were used in citrus plantations in China (Silva *et al*., 2006; Finkler, 2012).

"Entomopathogenic" refers to microorganisms capable of parasitizing and killing insects, using them as hosts for their development. In the case of fungi, their presence in insects has been recorded since 900 AD, but it was not until 1834 that Agostino Bassi demonstrated that *B. bassiana* could cause disease in silkworms, marking the beginning of the scientific use of these organisms in pest control (Roberts; Hajek, 1992). The official application of fungi in biological control began in 1879, with the use of *M. anisopliae* against root-feeding spittlebugs in Russia (De Faria; Wraight, 2007). Since then, various fungi have been studied, and beyond virulence, tolerance to environmental stresses such as heat, UV radiation, and low humidity has become a crucial criterion for selecting effective strains under real field conditions.

In addition to fungi, other entomopathogenic microorganisms such as viruses (Huger *et al*., 2005; Herrera-Vásquez *et al*., 2025), bacteria (Cossio-Bayugar *et al*., 2024), and nematodes (Machado *et al*., 2016; Labaude; Griffin, 2018) can also be applied in biological control.

In Brazil, the first records date back to 1923, with the application of the same fungus (*M. anisopliae*) against sugarcane spittlebugs (Alves; Faria, 2010). Over the years, they have gained prominence in research and applications related to microbial control of arthropods, especially in integrated pest management programs. In agriculture, commercial products based on *B. bassiana* and *M. anisopliae* are widely used to control various agricultural pests, including borers, caterpillars, and stink bugs. These are applied in the form of conidial suspensions, dry powders, and oil-based emulsions, with efficacy consistently validated under field conditions (Inglis *et al*., 2001; Shah; Pell, 2003; Quesada-Moraga *et al*., 2024).

In recent decades, a key factor recognized for the success of biological control using entomopathogenic fungi is the "environmental competence" of the strains. This competence refers to the fungus's ability to persist and maintain virulence in the target environment, even under adverse conditions such as temperature fluctuations, UV-B radiation, and relative humidity (Quesada-Moraga *et al*., 2024). These factors can significantly affect fungal propagule viability, as well as infection and host mortality. Therefore, selecting environmentally competent strains, particularly those originating from climatic zones similar to the application site, is considered the best strategy to ensure efficacy and consistency of field formulations.

The bioprospecting of entomopathogenic fungi aims to identify strains with high virulence, adaptability to adverse environments, genetic stability, and good sporulation. Among the most studied genera are *Metarhizium*, *Beauveria*, *Isaria*, and *Lecanicillium*. These fungi exhibit wide phylogenetic diversity, conferring ecological versatility and potential to adapt to

different hosts and environmental conditions (Pérez-González *et al*., 2004; Maina *et al*., 2018). Furthermore, the success of microbial control using entomopathogenic fungi is directly related to their ability to persist in the target environment, which highlights the importance of aligning the geographical origin of strains with application sites, thereby promoting greater efficacy and agronomic safety (Quesada-Moraga *et al*., 2024).

Climate change has altered interactions between pests and their natural enemies, potentially reducing the efficacy of biological control. Factors such as rising temperatures, changes in humidity, and increased solar radiation contribute to fungal propagule inactivation, reduced virulence, and limited fungal infection periods in the environment (Quesada-Moraga *et al*., 2024). This reinforces the need to select fungal strains with greater tolerance to environmental stresses and adaptation to new climatic conditions.

1.2.2 Entomopathogenic fungi for *Rhipicephalus microplus* control

Entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* have shown effectiveness in controlling this tick in both laboratory studies and field trials (Camargo *et al*., 2016; Mesquita *et al*., 2020; Marciano *et al*., 2021; Alcalá-Gómez *et al*., 2024). Among the most studied genera, *Metarhizium* stands out for its flexible metabolism and high environmental adaptability, which enable its survival and activity in tropical and subtropical soils (Rangel *et al*., 2008; Ortiz-Urquiza; Keyhani, 2015). The *M. anisopliae* complex comprises several species, such as *Metarhizium robertsii*, *Metarhizium brunneum*, and *Metarhizium globosum*, some of which are generalists and others specialists (Bischoff *et al*., 2009; Mongkolsamrit *et al*., 2020). Studies have shown that Brazilian strains such as *Metarhizium alvesii* and *Metarhizium humberi* also exhibit significant acaricidal potential.

These microorganisms act through direct infection, germinating on the tick cuticle and releasing enzymes such as chitinases, proteases, and lipases that degrade the cuticular barrier, facilitating fungal penetration and leading to the parasite's death (Mau *et al*., 2018; Castro-Saines *et al*., 2024). In addition to this mechanical and enzymatic action, *Metarhizium* also produces secondary metabolites such as destruxins, which contribute to pathogenicity by affecting ionic homeostasis, protein phosphorylation, and the host's immune functions (Zimmermann, 2007; Pal *et al*., 2007). Field tests on pastures and infested cattle have demonstrated significant efficacy of fungal formulations, with larval control ranging from 68% to 100% depending on the season and the type of formulation used (Ojeda-Chi *et al*., 2010; Alonso-Díaz *et al*., 2007; Barbieri *et al*., 2023). In naturally infested cattle, *M. anisopliae*-based

formulations achieved up to 91% efficacy in corral trials and around 75% under field conditions (Camargo *et al*., 2016). The combination with chemical acaricides has also proven effective, exhibiting synergistic effects observed in mixed formulations (Webster *et al*., 2015).

Significant progress has been made in using granules containing *M. robertsii* microsclerotia, applied to pastures, which enable gradual conidial release, reducing the need for frequent reapplications and improving environmental persistence (Marciano *et al*., 2021). Furthermore, the development of more stable and effective formulations, such as those based on ionic gelation-based encapsulation using 2% and 3% sodium alginate, has proven effective in protecting fungal conidia from adverse environmental factors such as UV-B radiation and high temperatures, while also extending their shelf life at room temperature. Studies show that encapsulated conidia display higher tolerance to these conditions and maintain prolonged viability under environmental conditions (Meirelles *et al*., 2023). Despite these advances, challenges remain (Hernandez *et al*., 2020). The sensitivity of conidia to extreme environmental conditions, such as prolonged drought and intense UV radiation, remains a major obstacle. Additionally, the tolerance of some tick populations to the fungus may be linked to immune mechanisms and cuticle composition (Ment *et al*., 2012), requiring the selection of more virulent strains or those adapted to different tick populations. Another issue is the need to optimize formulations to enable large-scale production, reduce costs, and meet regulatory requirements (Vega *et al*., 2012; Brasil, 2023).

In the veterinary field, the use of entomopathogenic fungi still faces commercial and regulatory barriers. Despite proven efficacy in laboratory, semi-field, and infested animal bioassays, the lack of commercially registered products specifically intended for use in livestock systems limits their adoption. Furthermore, direct application to cattle requires formulations that ensure adherence, viability, and safety for the animal (Beys-da-Silva *et al*., 2020).

Future perspectives include the development of formulations combined with protective adjuvants, the use of nanoencapsulation techniques, the genomic improvement and selection of more virulent strains, and applied research using omics tools to better understand virulence factors (Castro-Saines *et al*., 2024). The integration of entomopathogenic fungi with management practices such as pasture rotation and selection of resistant animals may enhance outcomes, foster more sustainable livestock production and reduce dependence on chemical acaricides.

In the veterinary field, however, challenges remain regarding the development of

economically viable formulations. Trials with *M. anisopliae*-based products in cattle pastures have shown reductions in tick populations when applied repeatedly, particularly when combined with pasture rotation and selective acaricide use (Camargo *et al*., 2016; Marciano *et al*., 2021). Applied research will be essential to adapt agricultural formulations to livestock environments, addressing factors such as stability under high solar exposure, safety for animal hosts, and suitability for topical applications.

1.2.3 Biological control of *Noctua pronuba* and other Lepidoptera from the Noctuidae family

Interactions between Lepidoptera and fungi are diverse and context-dependent, encompassing mutualistic, commensal, and parasitic associations. Some lepidopteran larvae, classified as mycophagous species, are capable of feeding on fungal fruiting bodies and completing their life cycle on this substrate (Ding *et al*., 2020). Others contribute indirectly to fungal ecology by acting as dispersal agents, since fungal spores may adhere to their body surfaces and be transported to new habitats, facilitating fungal spread and colonization (Li *et al*., 2022).

Beyond these indirect or mutualistic roles, fungi can exert strong regulatory pressures on lepidopteran populations through entomopathogenic interactions. Genera such as *Cordyceps, Beauveria, Metarhizium*, and *Lecanicillium* include species capable of infecting larvae and pupae, often leading to host death and thereby functioning as biotic regulators of natural populations (Majchrowska-Safaryan; Tkaczuk, 2021; Koyuncu *et al*., 2025). Infections typically begin with spore adhesion to the insect cuticle, followed by germination, penetration of the integument, and multiplication within the hemolymph. Ultimately, the fungus consumes host resources, causing systemic collapse and culminating in sporulation on the cadaver surface (Wang *et al*., 2024). This infection strategy not only impacts host survival but also plays an evolutionary role by shaping host–pathogen coadaptation and influencing ecological balances among species (Karthi *et al*., 2024).

In addition to direct pathogenicity, fungi may also affect Lepidoptera physiology and behavior through the production of secondary metabolites. Compounds such as alkaloids, phenolics, terpenoids, polysaccharides, and mycotoxins (e.g., ochratoxin A, fumonisin B1, zearalenone) have been reported to impair larval development, suppress immune responses, reduce motility, and compromise metamorphosis (Azab *et al*., 2001; Fan *et al*., 2013; Berini *et al*., 2016). Volatile organic compounds released by some fungal species can also modulate

insect behavior, influencing feeding, mate-seeking, or oviposition, with responses ranging from attraction to repellency (Dwisandi *et al*., 2024; Karthi *et al*., 2024). Such effects reveal that fungal interactions extend beyond mortality to include chemical ecology mechanisms that regulate insect populations and their ecological interactions. Moreover, mycophagous Lepidoptera may act as carriers of mycotoxins into agricultural products, raising concerns about food safety and potential indirect risks to human and animal health (Goudarzi *et al*., 2023).

Biological control emerges as a promising alternative for managing *N. pronuba* and other noctuid moths. Studies with parasitoids such as *Copidosoma floridanum* and entomopathogenic nematodes like *Steinernema carpocapsae* demonstrated their efficiency in reducing larval populations with minimal environmental disturbance (Gómez *et al*., 2022). Agronomic practices, including crop rotation and residue management, further reduce host availability and oviposition sites. While few studies directly address the use of fungi against *N. pronuba*, investigations with related noctuid species suggest promising potential. For instance, *B. bassiana* and *M. anisopliae* significantly decreased survival and fecundity of *Spodoptera frugiperda* through foliar spraying and seed treatment. Additionally, these fungi were capable of colonizing maize plants endophytically, exerting negative effects on larval development (Altaf *et al*., 2023).

Further evidence highlights the efficacy of *M. rileyi* against noctuid pests such as and *Helicoverpa armigera*. This fungus can evade host immune defenses by producing hyphal bodies with low recognition by pattern recognition receptors (e.g., C-type lectins, β-1,3-glucan recognition proteins), thus enhancing infection success (Li *et al*., 2021). Advances in genetic engineering have also improved the performance of *M. anisopliae* strains by incorporating toxin genes such as LqqIT1, which accelerates host mortality and reduces the median lethal time in *S. litura* and *Aphis craccivora* (Kumar, 2023). Similar success has been reported with baculoviruses such as ChinNPV in *Chrysodeixis includens*, which significantly reduce foliar damage and larval density in the field, reinforcing their importance as species-specific biocontrol agents (Botelho *et al*., 2019).

The integration of entomopathogenic fungi into Integrated Pest Management (IPM) programs is highly advantageous, contributing to reduced dependence on chemical insecticides and lowering environmental impacts (Embrapa, 2021). Their compatibility with other natural enemies, including parasitoids and predators, further enhances their utility as part of multipronged strategies (Goettel *et al*., 1990). Moreover, these fungi possess immune evasion mechanisms, such as modifying cell wall components and producing immunosuppressive

toxins like destruxins, that increase their success against insect hosts resistant to chemical control (Pal *et al*., 2007; Li *et al*., 2021).

Therefore, despite the limited number of studies directly involving *N. pronuba*, evidence from related noctuid species strongly supports the potential of entomopathogenic fungi as sustainable management tools. Currently, fungal products represent only 1–2% of pesticides marketed worldwide (Marrone, 2007), but their share is expected to grow with increasing restrictions on synthetic chemicals. In agriculture, fungal biocontrol agents are being successfully incorporated into IPM programs for crops such as soybean and maize (Ahamed *et al*., 2024).

1.3 Infection mechanisms and immune responses of entomopathogenic fungi in arthropods

1.3.1 Mechanisms of action of entomopathogenic fungi

The infectious process of entomopathogenic fungi, such as *M. anisopliae* and *B. bassiana*, in arthropods begins with the adhesion of conidia to the host cuticle. This adhesion is facilitated by mucilaginous substances on the spore surface, such as the protein Mad1 and the hydrophobin Hyd, which help in attachment to cuticular lipids under ideal temperature and humidity conditions (Shah; Pell, 2003; Wang; St. Leger, 2007). After adhesion, conidial germination occurs, forming germ tubes and appressoria, structures that combine mechanical action and enzymatic secretion to penetrate the cuticle (Pal, St. Leger; Wu, 2007; Zimmermann, 2007). In *R. microplus*, studies show that appressorium formation can be inhibited by specific cuticular compounds such as melanin, delaying infection (Grizanova *et al*., 2019). These structures play a key role in active cuticle penetration, mediated by mechanical action and the secretion of hydrolytic enzymes, such as chitinases, proteases, and lipases (Pal, St. Leger; Wu, 2007).

In insects, the chitin- and protein-rich cuticle is targeted by fungal enzymes like Pr1 and Pr2 (proteases) and chitinases, which degrade the structural components (Schrank; Vainstein, 2010). This penetration occurs mainly through the intersegmental regions of the cuticle or areas with less sclerotization. In ticks, however, the more complex epicuticle, with additional wax layers, requires the combined action of lipases and esterases for penetration (Ment *et al*., 2010). Penetration can occur through cracks in the dorsal cuticle or at the anus and spiracles, areas with lower physical and chemical resistance (Kalsi *et al*., 2020; Mascarin *et al*., 2019). Once

the cuticular barrier is overcome, the fungus reaches the hemocoel, where it adapts to the hemolymphatic environment. In this environment, hyphae can differentiate into blastospore forms, structures without rigid cell walls and with reduced expression of β-1,3-glucan, a molecule recognized by the host immune system (Butt *et al*., 2016; Schrank; Vainstein, 2010). The morphological transition observed in entomopathogenic fungi allows not only immune evasion but also efficient multiplication within the hemocoel (Ortiz-Urquiza; Keyhani, 2013).

In ticks, such as *R. microplus*, studies suggest that the presence of lipoproteins and iron-binding proteins in the hemolymph may influence blastospore survival and differentiation, granting the fungus greater evasion and proliferation capacity (Samish *et al*., 2001). In larvae of Lepidoptera, such as *Noctua pronuba*, rapid proliferation of yeast-like forms appears to facilitate colonization of metabolically active tissues, such as muscles and the gut (Wallis; Sisterson, 2024). Internal colonization continues with fungal spread through various tissues, including Malpighian tubules and the muscular system, impairing the arthropod's vital functions. In *R. microplus*, studies show that fungal infection can affect excretion, locomotion, and blood intake, culminating in parasite death within a few days (Fernandes; Bittencourt, 2008). After the host's death, the fungus may emerge through the cuticle, forming conidiophores and new conidia, completing the cycle and allowing dissemination into the environment (Samish *et al*., 2001).

This process has been similarly described in other arthropods, such as Lepidoptera larvae, including *N. pronuba*, where entomopathogenic fungi have also demonstrated the ability to penetrate the cuticle, colonize the hemocoel, and compromise larval development (Wallis; Sisterson, 2024). In addition to mechanical and enzymatic actions, entomopathogenic fungi produce secondary metabolites that play a central role in pathogenesis. Among these compounds, destruxins stand out, cyclic depsipeptides that interfere with cellular signaling by opening calcium channels, inducing muscle paralysis, dysfunction of Malpighian tubules, and apoptosis in host tissues (Pal; St. Leger; Wu, 2007).

The ability of entomopathogenic fungi to combine multiple infection and evasion mechanisms, such as enzymatic penetration, toxin production, and adaptive morphologies, gives these microorganisms a high potential as biocontrol tools (Ortiz-Urquiza; Keyhani, 2015; Vega *et al*., 2012). However, the variability in host immune responses, influenced by factors such as microbiota, age, species, and nutritional state, highlights the importance of conducting specific tests for each host-fungus system before implementing field control strategies (Butt *et al.*, 2016; Mascarin; Jaronski, 2016).

The combination of physical, enzymatic, and biochemical mechanisms makes entomopathogenic fungi effective agents in arthropod control. However, these microorganisms face robust defenses from hosts, which trigger sophisticated innate immune mechanisms in an attempt to contain the infection.

1.3.2 Immune response of arthropods to fungal infection

Arthropods have an exclusively innate immune system, as they lack lymphocytes and antibodies and, therefore, do not develop acquired immunity (Marmaras; Lampropoulou, 2009). This limitation is compensated by a sophisticated set of mechanisms capable of recognizing and rapidly responding to the presence of pathogens, such as entomopathogenic fungi. The innate immune response can be divided into three main components: physical barriers, humoral mechanisms, and cellular mechanisms (Gillespie *et al*., 1997; Sterba *et al*., 2011; Tan *et al*., 2013). The first line of defense involves physical barriers, such as the cuticle and the gut and reproductive epithelia, which hinder the entry of pathogenic agents (Marmaras; Lampropoulou, 2009). Once these barriers are overcome, humoral and cellular mechanisms are quickly activated.

The cellular immune response is mediated by hemocytes circulating in the hemolymph, which participate in phagocytosis, nodulation, and encapsulation of pathogens (Sterba *et al*., 2011; Tan *et al*., 2013). The humoral response, on the other hand, includes the production of antimicrobial peptides (AMPs), reactive oxygen species (ROS), pathogen recognition soluble proteins, such as thioester-containing proteins (TEPs), and the activation of enzymatic cascades responsible for coagulation and melanization of the hemolymph (Vass; Nappi, 2001; Kopacek *et al*., 2010; Smith; Pal, 2014). These mechanisms are crucial in combating fungal infection, as fungi often penetrate the cuticle and must be neutralized quickly.

Activation of these components occurs through classical immune signaling pathways, such as Toll, Imd, and JAK/STAT (Hoffmann, 2003; Myllymäki *et al*., 2014), which recognize pathogen-associated molecular patterns (PAMPs), such as chitin and β-glucans from the fungal cell wall. Although well characterized in model insects, such as *Drosophila melanogaster*, these pathways are still poorly understood in ticks (Smith; Pal, 2014; Oliva Chávez *et al*., 2017).

In insects, receptors like PGRPs and βGRPs detect β-1,3-glucans and activate these pathways, leading to the production of AMPs (Lemaitre; Hoffmann, 2007; Butt *et al*., 2016). For example, *Cecropin A* from *Bombyx mori* has high antifungal activity against *B. bassiana*, with increased expression in hemocytes and adipose tissue after infection (Lu *et al*., 2016). In

Helicoverpa armigera, *M. rileyi* evaded the cellular immune response due to the low affinity of PRRs for its modified hyphal bodies (Li *et al*., 2021). In the containment of infection, plasmatocytes and granulocytes phagocytose circulating blastospores, although the efficacy of this phagocytosis can be reduced by camouflage or changes in the fungal cell wall (St. Leger, 2008; Schrank; Vainstein, 2010). In *Galleria mellonella* and *Lymantria dispar* larvae infected by *M. anisopliae*, nodules and encapsulation of hyphae were observed, though not always effective (Dubovskiy *et al*., 2013). Additionally, the use of genetically modified *M. anisopliae* with scorpion toxin increased its virulence, demonstrating the potential of genetic engineering strategies to overcome the immune response (Kumar *et al*., 2023).

Melanization, mediated by the phenoloxidase (PO) pathway, is another relevant mechanism, promoting the confinement of fungal structures in melanized capsules. However, entomopathogenic fungi produce immunosuppressive compounds, such as destruxins, oosporein, and cyclopeptides, that inhibit PO activity and hemocyte function, facilitating immune evasion (Pal; St. Leger; Wu, 2007).

Although *Trichoderma atroviride* is not classified as an entomopathogenic fungus, its interactions with insects, particularly Lepidoptera, have been increasingly documented (Vinale *et al*., 2008). Unlike entomopathogenic fungi such as *Metarhizium* and *Beauveria*, *T. atroviride* is primarily recognized as a mycoparasite and plant growth-promoting agent (Harman, 2006). However, recent studies have revealed its ability to modulate insect immunity and influence host–fungus interactions in indirect ways (Chen *et al*., 2025).

Despite these documented effects on arthropod immune responses, the specific mechanisms by which *Trichoderma* modulates insect immunity remain largely unclear. Current evidence suggests that *Trichoderma* can suppress immune responses in Lepidopteran pests, as demonstrated in studies with *Trichoderma. asperellum* working synergistically with *B. bassiana* to suppress the immune response of *Ostrinia furnacalis* (Lepidoptera: Crambidae) larvae (Batool *et al*., 2020). Additionally, transgenic *Trichoderma* strains have been shown to influence bacterial community composition in the midgut of lepidopteran hosts, which may indirectly affect immune function and contribute to increased mortality (Li *et al*., 2013). However, unlike the well-characterized infection pathways of classical entomopathogens that involve direct cuticular penetration and subsequent immune evasion strategies (Ortiz-Urquiza and Keyhani, 2013; Singh *et al*., 2017), the immunomodulatory effects of *Trichoderma* appear to operate through alternative mechanisms that may include the production of bioactive secondary metabolites (Contreras-Cornejo *et al*., 2018; Ratnaweera *et al*., 2022) and indirect

effects on host physiology, rather than direct parasitic infection (Poveda, 2021).

In ticks, such as *Rhipicephalus microplus*, the immune response to fungal infection presents particularities. Phagocytosis by hemocytes is less efficient, and the production of antimicrobial molecules seems to depend on hemolymph proteins like lipoproteins and iron-binding proteins, which also affect fungal viability (Samish *et al*., 2004). Moreover, the gut microbiota modulates the systemic immune competence against infection (Kumar *et al*., 2021).

From a molecular perspective, ticks lack functional Toll and Imd pathways, with their defense being mainly mediated by the JAK/STAT pathway, lectin-like proteins, and complement-like components, such as IrC3s and IrA2Ms. These act as opsonins, facilitating phagocytosis of conidia (Fogaça *et al*., 2021; Fiorotti *et al*., 2022). Inhibition of these components increases mortality against *Metarhizium robertsii*, highlighting their importance in antifungal defense.

The intensity and effectiveness of the immune response vary depending on species, developmental stage, associated microbiota, and previous exposure history. Prior sublethal exposures can induce immune priming, resulting in more robust and specific responses (Fogaça *et al*., 2021). This phenomenon, similar to vertebrate immune memory, has been described in cockroaches and can be passed on to offspring (Bozler *et al*., 2020) and and discussed in several insect groups (Meunier, 2015). Although less studied in ticks, there is evidence that immunization with lipid molecules may modulate responses to pathogens like *A. phagocytophilum*, suggesting persistent defense mechanisms in these arachnids as well (Shaw *et al*., 2017).

Additionally, studies with insects show that activation of humoral components, such as AMPs and ROS in the gut, not only helps in pathogen containment but also directly influences the composition of the gut microbiota, promoting a balanced and functional environment (Khan *et al*., 2023). Thus, although the primary focus of the immune response is combating fungal infection, its repercussions can extend to the modulation of the symbiotic microbial community, which is particularly relevant in contexts of gut infection or oral exposure to entomopathogenic fungi.

1.4 Gut tick bacteriome: a complex and dynamic ecosystem

The microbiota of ticks comprises a diverse community of microorganisms, including bacteria, fungi, and viruses, that play crucial roles in the life cycle of these ectoparasites. These microorganisms influence development, reproduction, survival in different environments, and

the vector competence of ticks (Narasimhan; Fikrig, 2015). Over the years of research on this topic, several tick bacterial endosymbionts with commensal, mutualistic, or parasitic interactions have been identified (Sacchi *et al*., 2004; Scoles, 2004). Advances in molecular and sequencing techniques have allowed for a more detailed analysis of the microbiota in different tissues of ticks, such as salivary glands (Qiui *et al*., 2014), the gut (Narasimhan *et al*., 2014), ovaries, and Malpighian tubules. Studies have identified predominant bacterial genera in the tick microbiome, such as (Figure 3). The genus *Rickettsia* is particularly prevalent in various tissues, including the gut, salivary glands, and ovaries (Narasimhan; Fikrig, 2015). Additionally, species of *Arsenophonus* have often been identified in ticks, although their specific tissue distribution is not yet fully understood.

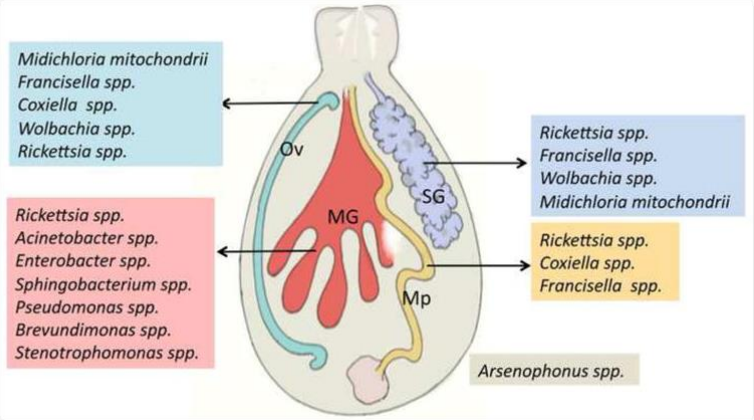

Figure 3: Predominate bacterial members of the tick microbiome (Narasimhan; Fikrig, 2015).

The gut stands out as a crucial environment, not only because it is the first point of contact between the tick and pathogens acquired during blood feeding, but also because it harbors a dynamic microbiota that directly influences the vector's physiology and its competence to transmit diseases. The gut microbiota, composed of a diverse community of microorganisms, performs vital functions such as assisting the tick in blood digestion, protecting against pathogens, and possibly modulating the immune response (Khan *et al*., 2023).

Blood processing in the tick gut begins with the ingestion of large volumes of fluid and blood cells, followed by the lysis of red blood cells in an alkaline gut lumen, mediated by digestive enzymes that release hemoglobin (Sodja *et al*., 2013). This hemoglobin is absorbed by digestive cells and broken down within acidic vesicles (pH 3.5–4.5) by hemoglobinases,

generating peptides, amino acids, and heme, which are either used as nutrients or eliminated through feces (Sodja *et al*., 2013). During this process, the gut microbiota, composed of microorganisms such as *Enterobacteria* and *Coxiella*, adapts to the heme-rich and reactive oxygen species (ROS) environment. Furthermore, the formation of a peritrophic matrix (PM) 6 to 12 hours after feeding separates the lumen from the epithelial cells, influencing interactions between luminal bacteria and the tick immune system, which can modulate colonization by pathogens such as *Borrelia burgdorferi* and *Babesia microti* (Kopacek *et al*., 2010; Smith; Pal, 2014).

Andreotti *et al*. (2011) conducted the first comprehensive study on the gut microbiota of *Rhipicephalus microplus*, identifying 11 bacterial genera, with a predominance of *Anaerobiospirillum* (29.5%) and *Brachybacterium* (21.9%), followed by *Papillibacter* (14.6%), *Enterococcus* (11.4%), *Borrelia* (7.9%), *Finegoldia* (4.7%), *Propionibacterium* (2.6%), *Succinivibrio* (2.0%), *Escherichia* (1.5%), and *Corynebacterium* (0.3%). The study also reported the presence of *Staphylococcus aureus* (3.5%) in the guts of adult females, as well as in adult males and eggs. Molecular analyses enabled the detection of fastidious bacteria, highlighting the potential role of *R. microplus* as a vector capable of transmitting microbes among vertebrate hosts, which may influence ecological and evolutionary aspects of its natural history.

Understanding the composition and diversity of the gut microbiota in *R. microplus* is essential to elucidate the interactions between ticks and their symbiotic microorganisms. These insights may lead to innovative tick-borne disease control strategies based on microbiota manipulation. Uncovering the functional consequences of tick–microbiome interactions is fundamental for developing new microbiome-based paradigms for controlling ticks and the pathogens they transmit. Zhong *et al*. (2007) demonstrated that curing *Amblyomma* ticks of their endosymbionts resulted in delayed oviposition, reduced egg hatchability, and decreased larval survival. In contrast, curing *Ixodes pacificus* of its rickettsial endosymbiont had no apparent impact on oviposition or tick survival.

Despite the continued use of whole ticks in many studies, this represents a major limitation, as it prevents accurate discrimination of tissue-specific microbiomes, such as those of the gut, from bacteria present on the exoskeleton or derived from environmental contamination. Studies involving dissected tissues, such as the gut, are essential for obtaining more robust functional inferences about host–microbiota interactions.

The introduction of techniques such as DNA metabarcoding has helped overcome some

of these limitations by combining molecular taxonomy with high-throughput sequencing. This approach enables the detection of uncultivable bacteria by clustering sequences into Operational Taxonomic Units (OTUs), which are taxonomically assigned based on database comparisons. As a result, it has become feasible to more precisely map the structure and function of microbial communities associated with different tick tissues, including the gut.

1.4.1 Role of the gut bacteriome in tick physiology and vector competence

The gut bacteriome of *Rhipicephalus microplus* plays a fundamental role in tick physiology and in its competence as a vector of pathogens. Several endosymbiotic bacteria, such as *Coxiella*-like, *Francisella*-like, and *Rickettsia*-like organisms, are vertically transmitted via the transovarial route and share genetic similarities with pathogens transmitted by these ectoparasites (Weller *et al*., 1998). It is believed that the initial acquisition of these bacteria occurred during tick feeding on infected hosts. Over time, these bacteria may have adapted to the tick's microbiota, persisting in its tissues or evolving from commensals into pathogens in vertebrate hosts (Noda *et al*., 1997). Studies suggest that these commensal bacteria provide protection to the tick by preventing other infections (de la Fuente *et al*., 2003; Ahantarig *et al*., 2013).

This symbiotic relationship has been explored as a potential strategy for controlling ticks and the diseases they transmit. The tick microbiota is composed of a diversity of microorganisms that interact in complex ways, influencing the vector's biology and its ability to transmit pathogens (Cabezas-Cruz *et al*., 2017; Becker *et al*., 2021; Bonnet; Pollet, 2021). The presence of *A. marginale* in the microbiome of *R. microplus* has been the focus of studies due to its relevance in bovine anaplasmosis. It has been observed that infection with *A. marginale* can modulate the composition of the tick's gut microbiota, influencing pathogen transmission (Guizzo *et al*., 2022). The presence of symbionts, such as *Coxiella*-like endosymbionts, can affect the survival and replication of *A. marginale* within the tick. Moreover, this interaction may modulate the expression of antimicrobial peptides in the gut, such as defensins and ixodidins, while others, such as *R. microplus* in, do not appear to respond in the same way to infection (Piloto-Sardiñas *et al*., 2024). Studies have shown that infection with *A. marginale* is associated with changes in the microbial diversity of the tick, with a reduction in bacterial diversity and shifts in the relative abundance of certain taxa. These alterations may impact the vector competence of the tick, making it more or less efficient in

pathogen transmission. Manipulation of the microbiota, through antibiotics, probiotics, or genetic strategies, has been proposed as a way to reduce this competence by modulating the immune response or competing with pathogens (Dennison *et al*., 2014).

Furthermore, the integrity of the peritrophic matrix (PM) in the gut is directly linked to the presence of a functional microbiota. In the study by Narasimhan *et al*. (2014), ticks treated with gentamicin or raised in sterile environments showed impaired PM formation, which hindered colonization by *Borrelia burgdorferi*. This observation reinforces the role of the gut microbiota not only in maintaining gut epithelial homeostasis but also in facilitating or blocking pathogen transmission. Changes in microbial composition can also modulate immune pathways such as Toll and Imd, influencing infection efficiency and vector competence (Zhong *et al*., 2007).

Understanding the interactions between the gut microbiota of *R. microplus* and the pathogens it transmits is essential for developing new strategies to control ticks and associated diseases. Manipulating the tick's microbiota, through genetic interventions, introduction of beneficial microorganisms, or antibiotic use, may offer novel approaches to reduce vector competence and control the spread of tick-borne pathogens (Cabezas-Cruz *et al*., 2017; Becker *et al*., 2021).

1.5 *Anaplasma marginale:* pathogen of veterinary significance

1.5.1 Biology and life cycle of *Anaplasma marginale*

A. marginale (Rickettsiales: Anaplasmataceae) (Dumler *et al*., 2000; Kocan *et al*., 2000) is a species of obligate intracellular Gram-negative bacterium. After infection, it invades the erythrocytes of cattle, which are subsequently phagocytosed by the mononuclear phagocytic system, forming inclusion bodies called morulae (24 to 48 hours after transmission) (Ribeiro; Lima, 1996). As it infects other erythrocytes, parasitemia increases significantly, leading to the acute phase of the disease (7 to 14 days after infection), during which the erythrocyte infection rate reaches about 70% (Richey, 1981), with symptoms such as fever, anemia, jaundice, and weight loss. In severe cases, it can lead to death, especially in young or immunosuppressed animals. In animals that overcome this phase, the infection stabilizes, the pathogen persists, but the number of infected erythrocytes decreases. The chronic phase of the infection can last from months to years, depending on the host's immune response and the occurrence of reinfections (Ribeiro; Lima, 1996). The ability of *A. marginale* to suppress the host's immune system and persist in the organism is one of the main challenges in controlling the disease. Infected cattle

remain as carriers and reservoirs (Kocan *et al*., 2010), posing a risk to other susceptible animals in the herd (Lopes *et al*., 2013).

1.5.2 Transmission of *Anaplasma marginale* by *Rhipicephalus microplus*

Worldwide, 20 tick species have been described as biological vectors of *A. marginale*. In Brazil, the transmission of this bacterium to other cattle occurs mainly through the arthropod vector *Rhipicephalus microplus* (Aguirre *et al*., 1994; Kocan *et al*., 2004; 2010; Atif, 2015). The development of *A. marginale* in the tick vector involves replication in the midgut cells followed by pathogen migration from the gut to the salivary glands, from where it can be transmitted to new hosts during blood feeding, at which point the tick inoculates the pathogen into the bloodstream of the bovine (Kocan *et al*., 2004). Transovarial transmission, where the pathogen is passed on to the next tick generation, has also been documented, although it is less common (Kocan *et al*., 2004).

There has been much discussion about the role of male ticks. Unlike females, which remain fixed on the skin of a single bovine until the end of feeding, males may move from one individual to another (Panizza *et al*., 2022), thus potentially transmitting the pathogen to other animals (Kocan *et al*., 2004; Kocan *et al*., 2010), a process facilitated by the gregarious behavior of cattle. Transplacental transmission, in which fetuses are infected during gestation, is rarely observed but has been reported in newborn animals exhibiting typical signs of the disease. Authors suggest that this mode of transmission can occur in cows that are chronic carriers (Silva *et al*., 2013).

In addition to ticks, *Stomoxys calcitrans*, *Haematobia irritans*, and species of the genus *Tabanus* are hematophagous dipterans that can act in the mechanical transmission and dissemination of *A. marginale* (Aubry; Geale, 2011; Oliveira *et al*., 2011). These insects possess mouthparts adapted for piercing or cutting the host's skin, allowing access to blood. During feeding, their mouthparts can become contaminated with infected blood and, upon biting another animal, mechanically transfer the pathogen. This form of transmission is considered mechanical because the pathogen does not develop or multiply within the insect but is simply transferred from one host to another. The efficiency of this transmission varies according to the dipteran species and feeding conditions (Scoles *et al*., 2005; Oliveira *et al*., 2011).

However, recent studies have questioned the traditional view of purely mechanical transmission by dipterans. In Progreso (Morelos), a tick-free region in the municipality of Jiutepec, Mexico, *A. marginale* was identified in *S. calcitrans*, suggesting that this fly may

serve as an alternative vector (Bautista *et al*., 2018). In a more recent study by Araújo *et al*. (2021), specific nested PCR for *A. marginale* detected the bacterium in different organs of *S. calcitrans*, indicating that the pathogen may be capable of colonizing various tissues in the insect. Molecular biology tools provide valuable insights into understanding the true modes of pathogen dissemination and are essential for developing more effective control strategies. These findings suggest that the interaction between the insect and the pathogen may be more complex than previously thought. Iatrogenic transmission may also occur through contaminated fomites, such as needles or surgical instruments used in procedures like ear tagging, castration, and dehorning (Kessler, 2000; Pereira *et al*., 2011).

1.5.3 Current challenges and strategies for diagnosis, treatment and control of bovine anaplasmosis

For the diagnosis of bovine anaplasmosis, epidemiological information, clinical signs, and laboratory tests are evaluated (Costa *et al*., 2011). Blood smears are a low-cost test; however, their efficiency varies according to the stage of the disease, being more effective during the acute phase and with high parasitemia (Dória *et al*., 2016). Serological tests are widely used to detect the immune response against *A. marginale* (Madruga, 2000; Santos *et al*., 2016). Although they are practical and inexpensive, their main limitation is the inability to distinguish between active infection and prior exposure, as they detect only antibodies and do not provide information about the course of infection (Cavalcante, 2007). Additionally, antigenic similarity between *Anaplasma* species can hinder specific discrimination (Palmer, 1989; Dreher *et al*., 2005). In contrast, molecular methods such as PCR and its variations (nested-PCR, semi-nested PCR, qPCR) offer high sensitivity and specificity, allowing early detection of pathogen DNA and the identification of asymptomatic carriers (Torioni de Echaide *et al*., 1998; Figueroa *et al*., 1993; Terkawi *et al*., 201; Santos *et al*., 2016). qPCR, in particular, enables precise quantification of the target DNA, such as the *msp5* gene of *A. marginale*, and detection of RNA transcripts such as *msp2* in infected ticks (Löhr *et al*., 2002; Futse *et al*., 2003). These techniques are especially useful for studying major surface proteins (MSPs), which play a crucial role in the interaction between the pathogen and the host (Kocan *et al*., 2010; dos Santos *et al*., 2019). Despite their higher cost compared to serological tests, molecular methods provide detailed information about the infection, including parasite load and gene expression, making them essential for advanced research and accurate diagnosis.

Clinical treatment of bovine anaplasmosis is essential for controlling outbreaks and

reducing the economic losses associated with the disease. Tetracyclines, especially oxytetracycline (OTC), are the most commonly used antibiotics for treating acute anaplasmosis. In Brazil, intravenous administration of OTC (11 mg/kg) for 3 to 5 days or long-acting formulations (20 mg/kg) every 72 hours are common practices (Alberton *et al*., 2015). However, recent studies show that the currently approved OTC doses (19.8 mg/kg subcutaneously at 3-week intervals) are not effective in fully eliminating *A. marginale* infection in chronically infected cattle. OTC temporarily reduced bacteremia, but infection levels returned to pre-treatment values after a few weeks, indicating that complete elimination of the pathogen is not achieved with current dosages (Curtis *et al*., 2021). Chlortetracycline, administered orally at a dose of 1.1 mg/kg for 60 days, also had no significant effect on reducing bacteremia, suggesting that higher doses or different regimens may be necessary to eliminate infection (Curtis *et al*., 2021). In addition to tetracyclines, enrofloxacin, a broad-spectrum antibiotic, has received conditional approval for treating clinical anaplasmosis, but its effectiveness in completely eliminating the infection still requires further investigation (Curtis *et al*., 2021; Facury-Filho *et al*., 2021). Another option used in Brazil is imidocarb dipropionate, administered as a single subcutaneous dose of 2.5 mg/kg, which has shown success in treatment, although its effectiveness may vary depending on the stage of infection. In severe cases, supportive treatment including fluid therapy and blood transfusions may be necessary to combat hemolytic anemia and other complications. Although these treatments are effective in reducing clinical symptoms, complete pathogen elimination remains a challenge, especially in endemic areas where reinfection is common.

Controlling the vector tick population *Rhipicephalus microplus* is one of the main preventive measures against bovine anaplasmosis. However, the traditional method of using chemical acaricides has limitations, such as the development of resistance and environmental impact (Guerrero *et al*., 2014). Alternative methods such as pasture rotation are widely used in the field and have shown effectiveness in both sustainable management and parasite control (Giles *et al*., 2017; Cruz-Gonzales *et al*., 2023). Additionally, promising strategies such as the use of nematodes, bacteria, and entomopathogenic fungi in biological control have been investigated and may become important tools in the future (Fernandes *et al*., 2012). It is important to highlight that maintaining endemic stability in a given area requires preserving a minimum vector population to ensure that animals are continuously immunologically challenged and maintain an effective immune response against the pathogen (Smith *et al*., 2000). Chemoprophylaxis is a strategy used on farms to control subclinical anaplasmosis,

prevent outbreaks, and reduce parasitemia. In Brazil, subtherapeutic doses of oxytetracycline or imidocarb have been employed to keep the infection under control, especially in endemic areas. However, continuous use of these drugs has raised concerns about the development of bacterial resistance, both in *A. marginale* and in other bacteria present in the herd (Ribeiro; Passos, 2002; Kocan *et al*., 2021). Recent studies have explored alternatives to traditional chemoprophylaxis, such as the use of probiotics and immunomodulators, which can reduce antibiotic dependence and minimize resistance risks. Furthermore, continuous antibiotic use may negatively affect the animals' microbiome and the environment. Therefore, chemoprophylaxis should be used with caution, preferably in combination with other control measures such as integrated vector management and vaccination to ensure long-term sustainability.

Vaccination is one of the most promising strategies for controlling bovine anaplasmosis, especially in endemic regions. In Brazil, live attenuated *A. centrale* vaccines are used to induce cross-immunity against *A. marginale*, although they do not prevent infection, only reduce the severity of clinical symptoms (Santos *et al*., 2019; Kocan *et al*., 2021). Studies have shown that vaccination with *A. centrale* can mitigate the disease, but there are reports of failures in inducing immunity and even outbreaks associated with its use (Salinas-Estrella *et al*., 2022). Ongoing research is exploring the development of recombinant vaccines based on specific antigens such as MSPs (Major Surface Proteins), although the antigenic diversity of *A. marginale* and the variability of MSPs pose significant challenges to developing a universally effective vaccine (Salinas-Estrella *et al*., 2022). Vaccination may be more effective in young cattle, but logistical issues such as distribution and the need for refrigeration remain challenges for large-scale implementation, particularly in remote areas (Salinas-Estrella *et al*., 2022).

1.5.4 Bovine anaplasmosis and its impact on animal health and productivity

Bovine anaplasmosis represents a significant challenge for both Brazilian and global livestock farming, with direct impacts on animal health and herd productivity, estimated at around US$800 million annually (Kocan *et al*., 2003). These losses result from various factors, including animal mortality, especially during acute outbreaks, reduced meat and milk production, and the costs associated with treatment and disease control.

In Brazil, where the disease is endemic, prevalence rates vary significantly across regions. In the Northeast, the molecular prevalence of *A. marginale* is 16.3% in the semi-arid

zone of Sergipe (Oliveira *et al*., 1992), while states such as Bahia and Paraíba report rates of 100% (Araújo *et al*., 1995; Madruga *et al*., 1994). This region is crucial for extensive cattle farming, particularly for beef production, with Bahia standing out as one of the largest beef producers in the Northeast. In the Southeast, São Paulo shows a prevalence of 98% (Andrade *et al*., 2004; Silva *et al*., 2016), while Rio de Janeiro and Minas Gerais report 100% (Ribeiro; Reis, 1981; Silva; Fonseca, 2014; Silva *et al*., 2015). The implementation of integrated strategies is essential to reduce losses and improve the sustainability of livestock farming in these regions.

Recent studies in India highlight those cumulative economic losses due to tick-borne diseases, including anaplasmosis, amount to US$787.63 million annually, with 52.15% of those losses attributed to reduced milk production (Singh *et al*., 2022). In Brazil, the resistance of *Rhipicephalus microplus* ticks to acaricides is a major challenge, with studies showing that resistant populations are widespread throughout the country, increasing production costs and reducing the effectiveness of control measures (Klafk *et al*., 2024).

Treatment costs for anaplasmosis are also significant. The use of antibiotics such as oxytetracycline and enrofloxacin is common, but complete pathogen elimination is difficult, especially in chronic cases. Moreover, supportive treatments, including fluid therapy and blood transfusions, are often necessary in severe cases, further increasing expenses (Mosqueda *et al*., 2012). In the United States, the average cost of a clinical case of anaplasmosis is estimated at US$660 per animal, with death losses accounting for 66% of that cost (Railey; Marsh, 2021). This may vary depending on the region and control strategy adopted, emphasizing the importance of preventive measures and early diagnosis. The presence of *A. marginale* in the herd is also associated with an increased incidence of other tick-borne diseases, such as babesiosis, due to coinfection with *Babesia bovis* and *B. bigemina*. This pathogen interaction can exacerbate clinical symptoms and increase economic losses (Gonçalves, 2000).

Infection with *A. marginale* causes severe anemia, weight loss, decreased milk production, and increased mortality, especially in young animals and during acute outbreaks. Animals that survive acute infection may become chronic carriers, maintaining low levels of parasitemia and serving as reservoirs for pathogen transmission. This chronic infection can lead to long-term productivity losses, negatively affecting herd profitability (Kocan *et al*., 2010).

In India, milk production loss due to anaplasmosis was estimated at 59.22 liters per cow per lactation, with significant economic impact on the dairy industry (Singh *et al*., 2022). In addition, coinfection with other tick-borne pathogens such as *Babesia bovis* and *B. bigemina*

can exacerbate clinical signs and increase economic losses (Gonçalves, 2000). In the United States, early detection of anaplasmosis through diagnostic tests such as competitive ELISA (cELISA) has been recommended as a cost-effective strategy to reduce economic losses, especially in regions where the disease is not endemic (Railey; Marsh, 2021).

New studies are essential to understand how a pathogen like *A. marginale* can affect a country's economy, particularly in regions where livestock is a crucial economic activity. Furthermore, it is vital to direct research efforts toward improving our understanding of disease transmission dynamics and vector competence. This approach could lead to the development of new strategies for controlling the tick vector, which may reduce the number of bovine anaplasmosis cases and other tick-borne pathogens, thereby lowering treatment and prevention costs.

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CHAPTER I

GUT BACTERIOME MODULATION IN *Rhipicephalus microplus* BY *Metarhizium anisopliae* UNDER *Anaplasma marginale* INFECTION

ABSTRACT

Ticks are obligate hematophagous ectoparasites of major veterinary importance, acting as vectors of several pathogens that severely impact livestock health and productivity. Among them, *Rhipicephalus microplus* stands out as the most harmful cattle tick in tropical and subtropical regions, mainly due to its ability to transmit *Anaplasma marginale*, the causative agent of bovine anaplasmosis. Understanding how bacterial communities within the tick gut respond to infection and biological control strategies is essential for clarifying vector competence. Entomopathogenic fungi such as *Metarhizium anisopliae* have been explored as biocontrol agents; however, little is known about how fungal infection interacts with tick bacteriota, particularly in the presence of pathogens. The present study explored, for the first time, changes in the gut bacteriome of *Rhipicephalus microplus* challenged with *Metarhizium anisopliae* and infected with *Anaplasma marginale,* using a metabarcoding approach considering V3-V4 region of the bacterial 16S rRNA gene. Experimental groups were established under four conditions: guts from *Metarhizium*-untreated females, either uninfected (CTR-) or infected with *Anaplasma* (CTR+); and guts from *Metarhizium*-treated females, either uninfected (Ma-) or infected with *Anaplasma* (Ma+). A total of 698 ASVs were identified, with 28.2% shared across all groups. The gut microbiota maintained a stable taxonomic composition, with Bacteroidota and Bacillota as the dominant phyla, and no significant differences in alpha diversity between the groups. However, according to the biomarker and co-occurrence network analyses, simultaneous exposure (Ma+) reshaped bacteriome, increasing the number of associated taxa with a distinct microbial composition. The Ma+ network was highly connected, predominantly by negative edge correlations, and exhibited greater stability even after node removal. This structural reorganization may reflect a compensatory response of the microbiota and host immune system to dual infection, rather than a sign of benefit. Functional prediction based on ASV phylogenetic placement (KEGG orthologs) indicated relatively conserved metabolic profiles, but the presence of the *Metarhizium* and *Anaplasma* increased functional diversity and the abundance of specific genes, suggesting microbial adaptation to support host or pathogen survival under fungal stress. Our findings revealed that while the gut bacteriome of this *R. microplus* population maintained a relatively stable taxonomic profiles under different experimental conditions, *A. marginale* infection appeared to enhance functional potential, highlighting complex host–pathogen–microbe interactions.

Keywords: tick-borne pathogens; entomopathogenic fungi; functional composition; network modulation

1 INTRODUCTION

The cattle tick, *Rhipicephalus microplus* (Canestrini, 1887), acts both as a debilitating hematophagous ectoparasite and a vector of hemoparasites (de la Fuente *et al*., 2008; Barré; Uilenberg, 2010). Its combined effects, direct parasitism and pathogen transmission, result in annual livestock losses of US$ 3.24 billion (Grisi *et al*., 2014). The tick's exceptional vectorial capacity is closely linked to its evolution as a parasite of vertebrates. Studies suggest that the relationship between ticks and pathogens is more complex than a simple parasitic interaction, with coevolution leading to mutual tolerance that is adapted to ticks' physiological and immunological traits. For example, O'Neal *et al*. (2021) reported that *Anaplasma* infections may actually enhance tick survival, such as by increasing cold tolerance, indicating that this coevolution benefits the vector and makes the interaction more adaptive and less harmful.

Among the pathogens vectored by *R. microplus*, *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) (Dumler *et al*., 2001) is a gram-negative obligate intracellular bacterium that infects ticks during blood feeding on infected cattle, causing bovine anaplasmosis and is one of the agents responsible for cattle tick fever (Albry; Geale, 2011; Puentes; Riet-Correa, 2023). When *A. marginale* is acquired from an infected host, several tissue barriers must be overcome within the tick body, such as the midgut, hemocoel, salivary gland, and ovary (transovarial transmission) (Hajdušek *et al*., 2013; de la Fournière *et al*.,2023;). Each of these tick tissues has microbial communities comprising pathogens, symbionts, and commensals that interact in a macrosystem in which the host is also inserted (Berg *et al*., 2020; Bonnet; Pollet, 2021; Hernandez *et al*., 2023). Among these tissues, the midgut plays a pivotal role as the primary site of pathogen entry and initial colonization (Hajdušek *et al*., 2013), it acts as both a physical and immunological barrier, where innate immune responses attempt to eliminate invading microbes. However, some pathogens have evolved mechanisms to evade or suppress these defenses, allowing them to persist and disseminate to secondary tissues like the salivary glands, from which they are transmitted to a new host (Bonnet; Pollet, 2021).

Understanding these midgut-pathogen interactions is critical for identifying potential targets to block pathogen transmission at the vector level. The tick midgut is a key tissue for pathogen survival and proliferation (Bonnet; Pollet, 2021; Fogaça *et al*., 2021). The midgut microbiota can be influenced by environmental factors such as temperature, humidity, and geographic localization (Gurfield *et al*.,2017; Brinkerhoff *et al*., 2020), as well as by host-related factors, including bovine skin contact and blood intake from vertebrate hosts previously treated with antibiotics. These effects have been demonstrated in studies involving the direct

administration of antibiotics to ticks, such as tetracycline (Zhong *et al*., 2007; Guizzo *et al*., 2022) or blood intake from vertebrate hosts that were previously exposed to antibiotics such as gentamicin (Narasimhan *et al*., 2014) or tetracycline (Mesquita *et al*., 2023). Such interventions demonstrate that altering the microbial environment of the tick midgut can affect its vectorial capacity. Therefore, it is important to elucidate how this system responds to other external agents, such as entomopathogenic fungi, especially in ticks already infected with *A. marginale*. Although *Metarhizium anisopliae* infects ticks primarily through the cuticle, systemic colonization may lead to physiological and immunological changes before death occurs (Aw and Hue, 2017; Zhang *et al*., 2024). In experimental settings, it is possible to evaluate sublethal or early-stage fungal effects while the host is still alive and functional. Investigating these interactions in ticks co-exposed to *A. marginale* allows us to explore potential bacteriome shifts that could affect pathogen colonization, host immunity, or microbial competition. In other words, despite the coevolution between ticks and pathogens, the gut microbiota can affect tick vectorial competence acquisition, colonization, and transmission (Bonnet and Pollet, 2021; Aguilar-Díaz *et al*., 2022; Narasimhan *et al*., 2021).

Advanced sequencing techniques such as next-generation sequencing (NGS) enable the examination of vast quantities of DNA fragments using a high-capacity sequencing approach (Morozova *et al*., 2008; Greay *et al*., 2018). Andreotti *et al*. (2011) used this tool to characterize midgut microbiota diversity first. Recently, other authors have confirmed the variability of the *R. microplus* midgut microbiota according to the region: Colombia (Segura *et al*., 2020), Brazil (Guizzo *et al*., 2022) and China (Zhang *et al*., 2022). Piloto-Sardiñas *et al*. (2024) explored the temporal changes in the microbiome of *R. microplus* over two years, focusing on its interaction with *A. marginale*. These findings emphasize the significant influence of *A. marginale* on the tick microbial community. These insights highlight the complexity of host-pathogen-microbiota interactions and reinforce the importance of studying how external agents may disrupt or reshape this balance. One such agent is the entomopathogenic fungus *Metarhizium anisopliae*, increasingly explored as a biocontrol alternative to chemical acaricides.

Unfortunately, many tick-borne diseases, such as anaplasmosis, do not have satisfactory treatments and cannot be completely cured (Curtis *et al*., 2021). The economic impact of *A. marginale* in areas with high livestock production includes high morbidity and mortality in susceptible cattle herds, decreased productivity, and increased veterinary costs (Parola *et al*., 2005; de la Fuente *et al*., 2008; Kocan *et al*., 2010; Barré; Uilenberg, 2010). To reduce tick-borne diseases, it is necessary to reduce the abundance of ticks. The use of chemical acaricides

commonly used method for tick control, but it has led to the emergence of resistant populations and raised concerns about human and environmental health. Using entomopathogenic fungi has arisen as a promising alternative when seeking a safer and more sustainable method. Studies have demonstrated the effectiveness of entomopatogenic fungi in the biological control of *R. microplus* (Camargo *et al*., 2016; Mesquita *et al*., 2020; Marciano *et al*., 2021; Meirelles *et al*., 2023). Other studies have also reported the impact of these fungi on the tick immune response (Kurtti *et al*., 2008; Fiorotti *et al*., 2019; 2022; Corrêa *et al*., 2022; Bório *et al*., 2023).

Nevertheless, it remains unknown whether fungal infection alters the gut microbiota of ticks already infected with *A. marginale*, or whether such modulation could affect the acquisition, persistence, or transmission of this pathogen. This represents a key knowledge gap that must be addressed. Since *R. microplus* commonly vectors *A. marginale* (Futse *et al*., 2003; Martins *et al*., 2020), evaluating microbial interactions in this context is relevant for better understanding the dynamics between biological control agents and tick-borne pathogens. Understanding the impact of fungal treatments on the *R. microplus* gut bacteriome is essential for evaluating their efficacy and potential side effects in pathogen control strategies. Clarifying this tripartite interaction, fungus–tick–pathogen, could improve our understanding of vector competence and support the development of integrative strategies to reduce the incidence of bovine anaplasmosis. The present study aimed, for the first time, to explore the influence of *Anaplasma* and *M. anisopliae* on the diversity, composition, and functionality of gut microbiota communities. Here, different experimental groups were compared to provide a broader understanding of the role of entomopathogenic fungi in integrated tick management and its implications for host-pathogen-microbiota interactions.

2 MATERIAL AND METHODS

2.1. Study site description

The experimental studies were conducted at the Laboratory of Microbial Control of Arthropods of Veterinary Importance, W. O. NEITZ Parasitological Research Station of the Department of Animal Parasitology, Veterinary Institute, Federal Rural University of Rio de Janeiro (UFRRJ), Seropédica, RJ, Brazil. Molecular detection and quantification experiments using real-time PCR were performed at the Laboratory of Molecular Diagnostics and Poultry Health (LASAVE), Department of Epidemiology and Public Health, Veterinary Institute, UFRRJ, RJ, Brazil. Metabarcoding analyses were carried out at the Laboratory of Bioinformatics, Department of Agricultural Sciences, Livestock and Environmental Biotechnology, School of Agricultural and Veterinary Sciences, São Paulo State University (UNESP), Jaboticabal, RJ, Brazil.

2.2 Experimental design

The experimental design for the characterization of the *R. microplus* gut bacteriome involved several key steps, structured into four main phases (Figure 1):

- Tick Obtention and Treatment: Female *R. microplus* ticks, naturally fed on *Anaplasma marginale*-infected calf, were collected. These females were subsequently divided into two experimental groups: CTR [ticks topically treated with Tween 80® 0.01% (v/v)] and Ma (ticks topically treated with *M. anisopliae* suspension). All groups were incubated for 72 hours under controlled conditions.
- Dissection and DNA extraction: Following incubation, the guts of individual female ticks from two groups were carefully dissected. The gut tissues were then macerated, and total DNA was extracted, ensuring high quality and purity for subsequent molecular analyses.
- *Anaplasma* detection and fungal confirmation via qPCR: The extracted DNA was utilized for quantitative Polymerase Chain Reaction (qPCR) to detect and quantify the relative load of *Anaplasma marginale* (targeting the msp1β gene) and to confirm the presence of *Metarhizium anisopliae* (targeting the EF-1α gene) in the tick guts. The *R. microplus* 18S rRNA gene was used for normalization.
    - Following molecular confirmation of *A. marginale* and *M. anisopliae* infection via qPCR, the two groups were categorized into four distinct experimental groups: CTR-

(*Metarhizium*-untreated females, uninfected), CTR+ (*Metarhizium*-untreated females, infected with *Anaplasma)*, Ma- (*Metarhizium*-treated females, uninfected) and Ma+ (*Metarhizium*-treated females, infected *with Anaplasma*

- Bioinformatics and microbiome analysis: The gut bacteriome was characterized by 16S rRNA gene sequencing (V3-V4 regions) using the Illumina MiSeq platform. Raw reads underwent rigorous bioinformatics processing (quality control, trimming, merging, ASV inference, chimera removal, taxonomic classification using Greengenes2 and NCBI RefSeq, phylogenetic inference, and contaminant removal). Subsequently, advanced microbiome analyses were performed, including microbial ecology (alpha and beta diversity, biomarker detection via LEfSe), microbial structure (co-occurrence networks via SparCC, topological characteristics, robustness), and functional prediction (PICRUSt2 for KEGG orthologs). All statistical analyses (ANOVA on ranks, PERMANOVA) were conducted using appropriate R packages.

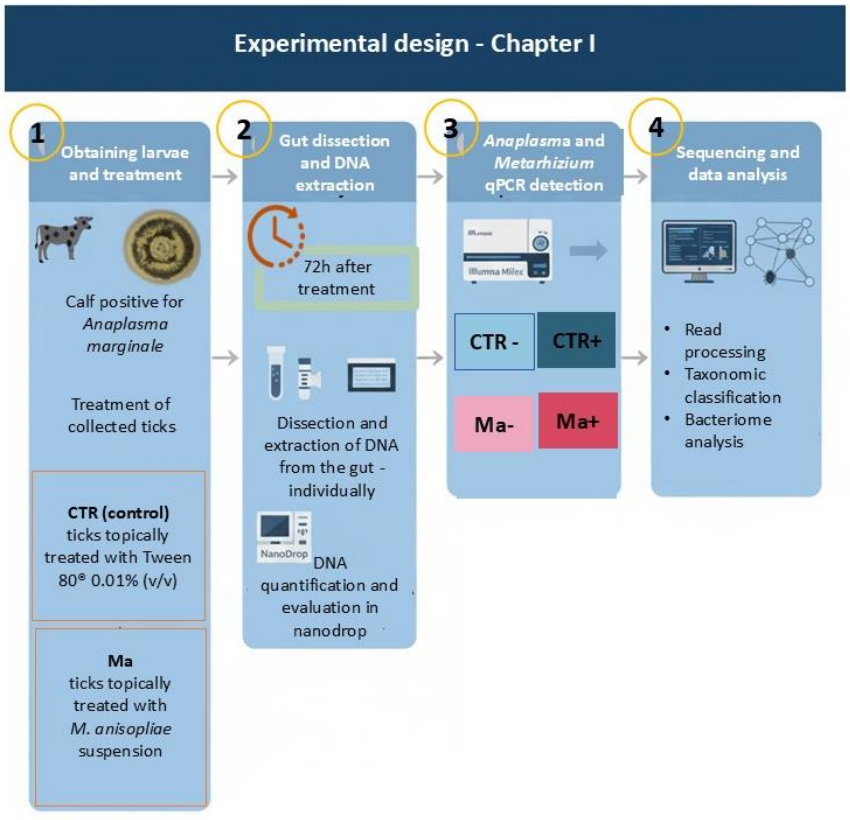

Figure 1: Experimental methodology design for Chapter 1. The scheme illustrates the main stages involved in characterizing the *Rhipicephalus microplus* gut bacteriome under dual infection with *Metarhizium anisopliae* and *Anaplasma marginale*.

2.3 Calf selection and tick collection

One calf naturally infected with *Anaplasma marginale*, confirmed by Nested PCR (nPCR) following the protocol of de Echaide *et al*. (1998) was selected for this study. Blood samples were collected from the jugular vein using sterile Vacutainer needles and EDTA-coated tubes (Becton Dickinson), and stored at 4 °C at the W.O. Neitz Parasitological Research Station at UFRRJ), Brazil (CEUA [Ethics Committee on the Use of Animals]/Veterinary Institute, UFRRJ; protocol nº. 4832020623 -Appendix 1). DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN), according to the manufacturer's instructions. One calf-positive was artificially infested with *R. microplus* larvae from a Laboratory of Microbial Control of Arthropods of Veterinary Importance – maintained colony, which was routinely tested to ensure the absence of common bovine hemoparasites such as *Babesia bovis, B. bigemina* and *A. marginale* (Porto Alegre strain) and held at the W.O. Neitz Parasitological Research Station at the (UFRRJ), Brazil (CEUA [Ethics Committee on the Use of Animals]/Veterinary Institute, UFRRJ-protocol nº. 6274250624 - Appendix 2). Female ticks were naturally fed on the calf for 21 days and then manually removed from the host.

2.4. *Metarhizium anisopliae* fungal suspension

Metarhizium anisopliae LCM S04 (Mesquita *et al*., 2020) was used in this study. This soil-borne Brazilian native isolate (Correa *et al*., 2022) was maintained in the Collection of Entomopathogenic Fungal Cultures of the Laboratory of Microbial Control of Arthropods (CCFELCM), UFRRJ, Seropédica, Brazil. The isolate was cultured on oat medium (60 g oat bran, 12.5 g agar in 1000 mL of distilled water) under controlled conditions (25 ± 1 °C; and relative humidity (RH) ≥ 80%). As the present study assessed Brazilian genetic heritage, the research was registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Sisgen) under the code AA47CB6.

After 14 days, the conidia were scraped from the culture medium and suspended in sterile distilled water solution containing 0.01 % (v/v) polyoxyethylene sorbitan monooleate-Tween 80® (Sigma-Aldrich). The fungal concentration was adjusted to 1×10^8 conidia. mL^{-1} using a hemocytometer. Conidial viability was determined by plating an aliquot of 1×10^5 conidia mL^{-1} suspension (20 µL) on potato dextrose agar (PDA), followed by incubation at 25 ± 1 °C and RH ≥ 80%. After 24 h, conidial germination was observed using optical microscopy (× 400) (Nikon, E200) after staining with methyl blue (Sigma-Aldrich). A minimum of 300 germinated and non-germinated conidia were evaluated, and the percentage

of germination was calculated (Lopes *et al*., 2013). Conidia were considered to have germinated when germ tubes were visible.

2.5. Fungal treatment in *Rhipicephalus microplus* females

Tick females were initially divided into two groups with 15 ticks each: CTR (control) (ticks topically treated with Tween 80® 0.01% (v/v); 20 µL) and Ma (ticks topically treated with 20 µL of *M. anisopliae* suspension at 1×10^8 conidia mL^{-1}). The treatment was applied to the dorsal region of the tick, and the ticks were kept at 25 ± 1 °C and RH ≥ 80%. Seventy-two hours after treatment, the guts of individual females from each group were dissected for DNA extraction.

2.6. *Rhipicephalus microplus* dissection and gut DNA extraction

The guts of *R. microplus* females were dissected using sterile tweezers and scalpel blades using iced sterile NaCl 0,9%. The removed gut tissues were washed twice in sterile saline solution and stored in RNA later (Thermo Fisher Scientific) at -80 °C until extraction. Each tick was dissected according to a protocol adapted from Tidwell *et al*. (2021). First, the guts were washed with 1 mL phosphate-buffered saline (PBS) and centrifuged for 5 min. This process was repeated thrice to completely remove the RNA later reagent. The tick guts were frozen in liquid nitrogen and macerated using sterile pestles. The DNA of the gut homogenate was extracted using the DNeasy Blood & Tissue kit (QIAGEN), according to the manufacturer's instructions.

2.7. Analysis of the DNA quality and presence of *Anaplasma marginale* in the experimental groups

The tick guts DNA concentration and purity were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The integrity of the total DNA was assessed by electrophoresis on a 1% agarose gel. The *A. marginale* DNA was detected using qPCR, which specifically targeted the gene from superficial protein 1-beta (*msp 1β*) from *A. marginale* amplification (Carelli *et al*., 2007) (Table 1). The reactions were performed in triplicate on the StepOnePlusTM Real-Time PCR System Applied Biosystems (Thermo Fisher Scientific), with a final reaction volume of 12 µL containing 6 µL of TaqMan Universal PCR Master Mix (Thermo Fisher Scientific), 0,5 µM of each primer, 0,25 µM of the probe, and 3 µL of total DNA at 30 ng/µL. Negative controls were prepared using molecular-grade water. A DNA

sample extracted from bovine blood naturally infected with *A. marginale* was used as positive control. The thermocycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 30 s, and 72 °C for 30 s. The endogenous gene of *R. microplus* was also amplified by qPCR to normalize the data and calculate the relative pathogen load, using *R. microplus* 18S ribosomal RNA gene (18S rRNA) (Saldivar *et al*., 2008) (Table 1). The reactions were carried out in triplicate in a final volume of 12 µL containing 6 µL of TaqMan Universal PCR Master Mix (Thermo Fisher Scientific), 0,9 µM of primer, 0,25 µM of the probe, and 3 µL of total DNA, completed with molecular-grade water. The thermocycling conditions consisted of an initial step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.8. Detection of *Metarhizium anisopliae* in the gut of *Rhipicephalus microplus* female

The same DNA samples used in section 2.5 (2.5 µL) were used to detect the presence of *M. anisopliae* through qPCR. The target gene encodes the superficial protein Elongation Factor 1-alpha (*EF-1α*) (Nijhof *et al*., 2009) (Table 1). The reactions were performed in triplicate with a final volume of 12 µL containing 6 µL of SYBR® PCR Master Mix System Applied Biosystems (Thermo Fisher Scientific) and 0.5 µM of each primer. Negative controls were prepared using molecular-grade water, and a DNA sample extracted from *M. anisopliae* cultured in oat medium served as the positive control. The thermocycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. This was followed by melt curve analysis that was configured with a temperature increment of 0.3 °C every 15 seconds, ranging from 60 °C to 95 °C.

Table 1. Primers and probes sequences used for quantitative PCR analyses

Target	Primer or probe[1]	Sequence (5'-3')
Anaplasma marginale	AM – F	TTGGCAAGGCAGCAGCTT
(*msp1 β*)	AM – R	TTCCGCGAGCATGTGCAT
Carelli *et al*., 2007	AM – Pb	6 FAM – TCGGTCTAACATCTCCAGGCTTTCAT - QSY
Rhipicephalus	Rm18SF	CCTGAGAAACGGCTACCACATC
microplus	Rm18SR	TGCCGGGAGTGGGTAATT
(*18S* rRNA)	Rm - Pb	VIC-AGGAAGGCAGCAGGCGCGC

Saldivar *et al*., 2008		
Metarhizium anisopliae Elongation Factor 1-alpha (*EF-1α*) Nijhof *et al*., 2009	ELF1A-F	CGTCTACAAGATTGGTGGCATT
	ELF1A-R	CTCAGTGGTCAGGTTGGCAG

[1] **F**: forward primer; **R**: reverse primer; **Pb**: probe

2.9. Study design

The *R. microplus* gut bacteriome was characterized by 16S rRNA gene sequencing. Experimental groups were established under four conditions: guts from *Metarhizium*-untreated females, either uninfected (CTR-) or infected with *Anaplasma* (CTR+); and guts from *Metarhizium*-treated females, either uninfected (Ma-) or infected with *Anaplasma* (Ma+). Tick guts testing positive for *Anaplasma* were assigned to the infected groups (+). All ticks exposed to *Metarhizium*, irrespective of fungal detection within the gut, were assigned to the treated groups. DNA extracted from individual tick gut samples was used to construct 16S rRNA V3–V4 region amplicon libraries (Abraham *et al*., 2017) and sequencing was carried out on the Illumina MiSeq platform (MiSeq Reagent Kit V2 Nano, 2 × 250 cycles).

2.10. Processing of raw reads

Raw reads were assessed for quality using FastQC software (Andrews, 2010). Primer sequences were trimmed using Atropos (Didion *et al*., 2017 with the "--discard-untrimmed" option to eliminate unprimed sequences. Reads with an average Phred quality score below 25 were excluded using the "--average_qual 25" parameter. Read pairs were merged using FLASH (Magoc; Salzberg, 2011) with default settings. The resulting high-quality merged reads were then processed using the DADA2 pipeline (Callahan *et al*., 2016) implemented in R (R Core Team, 2023). The "filterAndTrim" function was applied to remove sequences with more than four expected errors (maxEE = 4). The Amplicon Sequence Variants (ASV) were inferred for each sample, followed by filtering of chimeric sequences using the "removeBimeraDenovo" function to retain biologically correct sequences. The ASVs were taxonomically classified using the "assignTaxonomy" function of DADA2, referencing the "Greengenes2" database (McDonald *et al*., 2024). Additionally, ASVs were aligned with the NCBI RefSeq 16S rRNA database using BLAST (Altschul *et al*., 1990) for secondary verification. Phylogenetic

relationships among ASVs were inferred using the Neighbor-Joining algorithm with the NJ function in the phangorn package (Schliep, 2011). Statistical support for the phylogenetic tree was assessed using the "bootstrap" method with the "bootstrap.pml" function, employing 100 bootstrap replicates. Finally, potential contaminant sequences were identified and removed using the Decontam R package (Davis *et al*., 2018) "Blank" samples containing 0.9% NaCl, processed together with the experimental treatments, were included in the sequencing batch to detect contaminants. Decontam was run using the "prevalence" method with a detection limit of 0.5, and identified contaminant ASVs were excluded from further analyses.

2.11. Microbiome analysis

2.11.1. Microbial ecology

Sampling efficiency was assessed using rarefaction curves generated with the "amp_rarecurve" function of the R package, ampvis2 (Andersen *et al*., 2018). The samples were rarefied to the library with the lowest number of sequences (8,868 reads), and all subsequent analyses were performed based on rarefied data. The ASV counts and taxonomic annotations were exported in the phyloseq format (R package, phyloseq; McMurdie; Holmes, 2013). To standardize the dataset for microbiome analyses, the phyloseq object was transformed into compositional data using the "phyloseq_standardize_otu_abundance" function of the R package MetagMisc (Mikryukov, 2022). Alpha diversity was calculated using observed richness measures (Richness) and Pielou's evenness indices, using the "alpha" function of the R package microbiome (Lahti; Shetty, 2012) and Faith's phylogenetic diversity, using the "pd" function of the R package picante (Kembel *et al*., 2010). Beta diversity was analyzed by UniFrac distances (weighted) between samples using the "distance" function of the phyloseq. The beta dispersion of the samples within each treatment group was also assessed to evaluate the variation between groups using the "betadisper" function of the R package vegan (Oksanen *et al*., 2019). For biomarker detection, the Linear Discriminant Analysis Effect Size (LEfSe) approach (Chang *et al*., 2022) was used at each taxonomic level. This analysis was implemented using the MicrobiomeAnalyst web platform (Dhariwal *et al*., 2017), and the thresholds for significance were $p < 0.05$ and LDA > 1.5.

2.11.2 Microbial structure

Co-occurrence network analyses were performed using the "Sparse Correlations for

Compositional Data" (SparCC) method implemented through the R package SpiecEasi (Kurtz *et al*., 2015). Taxonomic data tables were collapsed to the taxonomic genus level and used to calculate the correlation matrix. Only relationships with correlation coefficients greater than 0.75 or less than -0.75 and with a p-value ≤ 0.05 were considered. Furthermore, to reduce noise and focus on relevant genera, only those with a summed relative abundance greater than 0.01% in the dataset were included. Co-occurrence networks were constructed and topological characteristics, such as degree of connection, average closeness, and betweenness centrality, were calculated using the R package igraph (Csardi; Nepusz, 2006). Robustness analysis of the networks, which consists of the evaluation of the stability of the networks after the removal of nodes by different criteria based on their topological characteristics, was performed using the R package, NetSwan (Lhomme, 2015).

2.11.3 Functional prediction

To infer the functional potential of the microbiomes, the PICRUSt2 program (Douglas *et al*., 2019) was used to predict the KEGG orthologs (KOs) in each sample from the ASVs. Using the predicted orthologs, functional richness and diversity were calculated using the "alpha" function of the Phyloseq package. Differentially abundant functions between conditions were identified using the DESeq2 methodology (R package DESeq2; Love *et al*., 2014), with significance thresholds set at $p < 0.05$ and |Log2 fold-change| > 1.

2.12. Statistical analysis

For general statistical comparisons, two-way ANOVA was used, considering the factors of treatment and *A. marginale* infection status based on ranked values ("ANOVA on ranks"), as proposed by Sawilowsky (1990). To evaluate the significant differences between the treatment means, a confidence level of 95% ($p < 0.05$) was adopted. For variables that presented significant differences, multiple comparisons were performed using Fisher's LSD post-hoc test using the R package, agricolae (Mendiburu, 2023). Differences in beta diversity between groups were evaluated using Permutational Multivariate Analysis of Variance (PERMANOVA) with the "adonis2" function of the R package vegan. Post-hoc pairwise comparisons were conducted using the "pairwise.adonis" function of the R package (Arbizu, 2017). Multidimensional distances were visualized using Principal Coordinate Analysis (PCoA). Graphical representations were created using the R package ggplot2 (Wickham 2016). Heatmaps of taxonomic and functional data were generated using the R package ComplexHeatmap (Gu *et*

al., 2016).

3 RESULTS

3.1. Molecular confirmation of infection by *Anaplasma marginale* and *Metarhizium anisopliae*

qPCR was performed to validate the infection status of the tick samples and detect *A. marginale* and *M. anisopliae*. The fungal suspensions used in the experiments had a conidia viability of at least 95%, ensuring the effectiveness of the treatment. Among the samples in the infected control group (CTR+), nine out of 15 gut samples were positive for *A. marginale*. In the group treated with *M. anisopliae* and infected with *A. marginale* (Ma+), six out of 15 samples were positive for *A. marginale*. In the groups treated with fungi only two gut samples from Ma+ tested positive for *M. anisopliae*. These molecular confirmations supported the validity of the experimental infection groups facilitated the interpretation of shifts in microbial diversity and functional traits, as described in the following sections.

3.2. The gut microbiota of *Rhipicephalus microplus* maintains bacterial community diversity and structure under *Metarhizium anisopliae* treatment and *Anaplasma marginale* infection

Analysis of the gut bacterial composition of ticks revealed 698 ASVs, of which 197 (28.22%) were common to all groups (Figure 2A). Only a small number of ASVs were unique to specific treatments, ranging from four ASVs (0.57%) in CTR- to 28 ASVs (4.01%) in CTR+, 24 ASVs (3.43%) in Ma-, and six ASVs (0.85%) in Ma+. PCoA analysis, based on the weighted UniFrac distance matrix, explained 51.77% of the variance observed in the first two axes. PERMANOVA results indicated no significant differences in the composition of gut microbial communities among the groups analyzed ($p > 0.05$) (Figure 2B). However, beta dispersion analysis revealed significant differences, with the Ma+ group showing less dispersion than the CTR+ group ($p = 0.014$). Alpha diversity was assessed using three metrics (Richness, Pielou's evenness, and Faith's phylogenetic diversity). No significant differences in alpha diversity were observed between the treatments ($p > 0.05$) (Figure 2C).

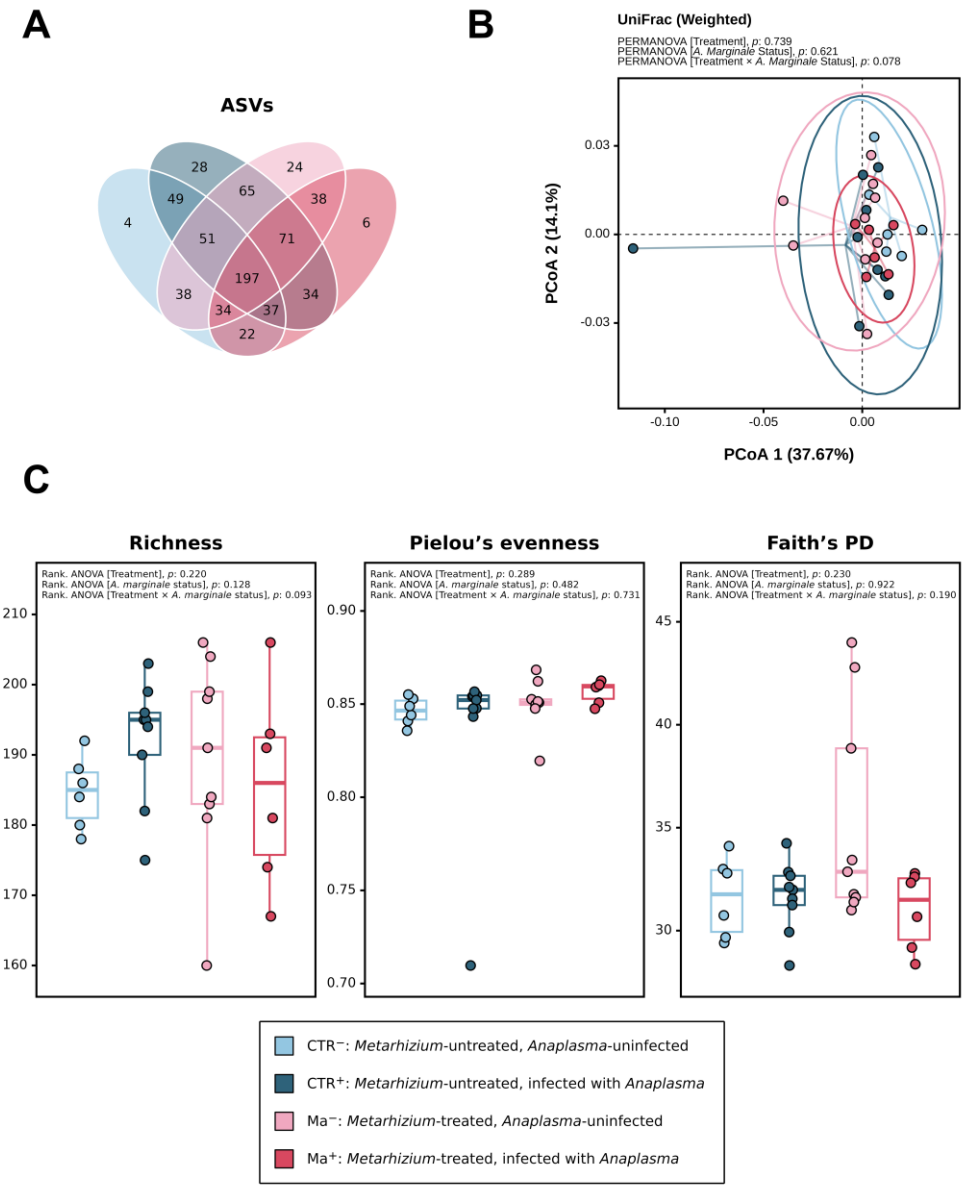

Figure 2. Shared and unique amplicon sequence variants (ASVs), microbial beta diversity, and alpha diversity of bacterial communities in *Rhipicephalus microplus* guts, comparing samples treated or untreated with *Metarhizium anisopliae* and infected or uninfected with *Anaplasma marginale* (**A**) Venn diagram displaying the distribution of shared and unique ASVs among treatment groups. (**B**) Microbial composition among samples by principal coordinate analysis (PCoA) of weighted UniFrac distances. Statistical significance was assessed using a two-way PERMANOVA ($p < 0.05$). Small circles represent the samples, and ellipses represent the dispersion of the samples. (**C**) Alpha diversity metrics, including ASV richness, Pielou's evenness, and Faith's phylogenetic diversity (Faith's PD), were compared using a two-way ANOVA on ranks ($p < 0.05$).

3.3. Shared taxa predominate in the gut microbiota of *Rhipicephalus microplus* across experimental groups treated with *Metarhizium anisopliae* and infected with *Anaplasma marginale*

Taxonomic assignment was highly effective, with 98.82% of sequences successfully classified at the genus level. Venn diagrams at the phylum and genus levels (Figure 3A) revealed substantial taxon sharing between the treatments. Of the 12 phyla identified, none were exclusive to a single treatment, and eight phyla (66.6%) were shared across all groups. At the genus level, 125 of the 234 taxa (53.4%) were shared among the groups, whereas only one genus (0.42%) was exclusive to the CTR- and Ma+ treatments, and seven genera (2.99%) were exclusive to the CTR+ and Ma- treatments. Phylum (Figure 3B) and family level profiles showed high similarity in relative abundance among the analyzed groups, with Bacteroidota and Bacillota being the most abundant phyla. The relative abundance of Bacteroidota varied from a mean relative abundance of 43.18% (CTR+) to 47.49% (CTR-), whereas Bacillota varied from 37.87% (Ma-) to 38.56% (CTR-). At the genus level, the relative abundance of the 50 most abundant genera (Figure 3C) did not reveal any specific sample grouping by treatment in the heatmap. The most abundant genera were Phocaeicola (14.21%), Prevotella (13.33%), and Bacteroides (6.17%), all of which belonged to the Bacteroidota phylum.

LefSe analysis (Figure 4) identified significant biomarkers, representing 3.15% of the total ASVs analyzed (698 ASVs). The following were detected: (CTR-) *Ruminococcus, Sutterella wadsworthensis, Faecousia, Pumilibacteraceae, Lachnospiraceae, Hyphomicrobium zavarzinii, Barnesiella* and *Agathobaculum butyriciproducens*; (CTR+) *Bradyrhizobium ottawense* and *Parasutterella gallistercosis*; (Ma-) *Bacteroides stercoris*, UBA5905 and *Faecalibacterium prausnitzii*; (Ma+) *Acetatifactor acetigignens, Pantoeae dispersa, Akkermansia muciniphila, Lepagella muris, Lachnospiraceae, Kineococcus vitellinus, Paramuribaculum* and *Bacteroidaceae.* All biomarkers were classified with high taxonomic accuracy, highlighting the clear distinctions between the groups analyzed.

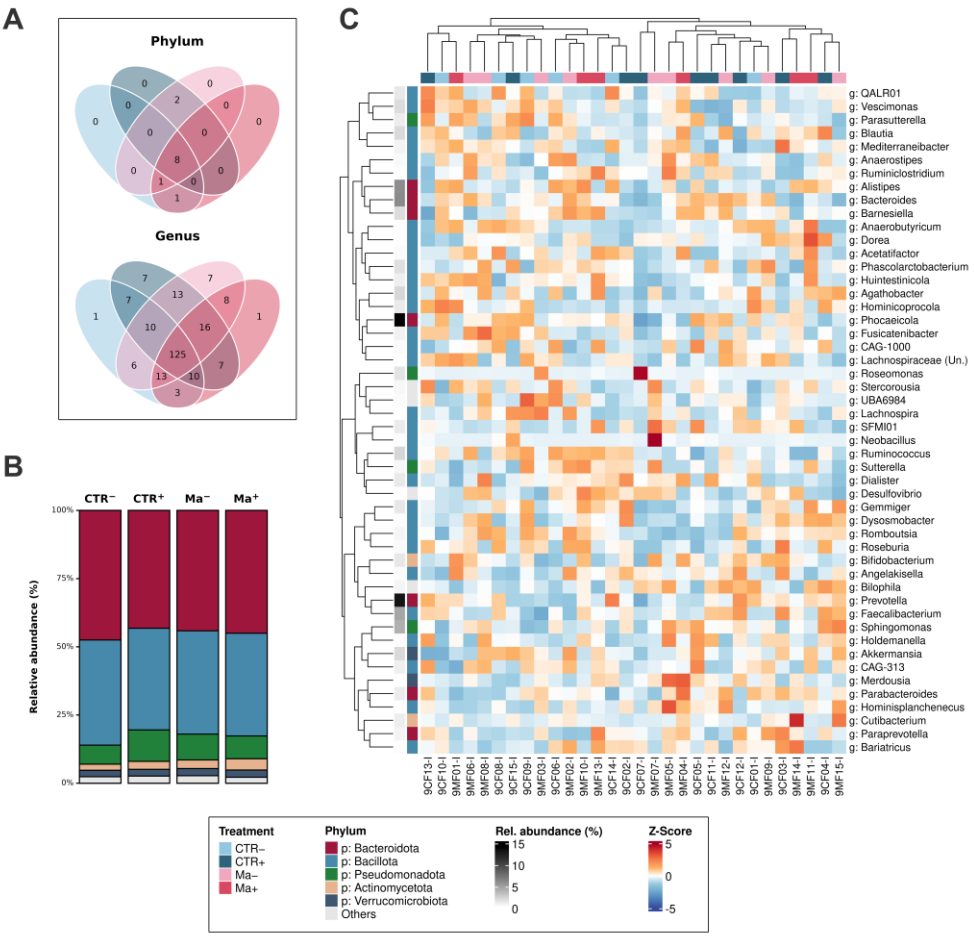

Figure 3: Bacterial taxonomic composition in *Rhipicephalus microplus* gut. (**A**) Venn diagrams illustrating the overlap of bacterial phyla and genera between the treatment groups (CTR-, CTR+, Ma-, and Ma+). (**B**) Bar graphs showing the relative abundance of bacterial phyla in the treatments. Low-abundance phyla were grouped under "Others." (**C**) Heatmap displaying the distribution of the 50 most abundant bacterial genera, with abundance transformed into Z-scores. The overall abundance and corresponding phyla are indicated in the left column of the graph. Sample treatments are represented by color-coded bars above the graph. Hierarchical clustering of rows and columns was performed using Ward's method.

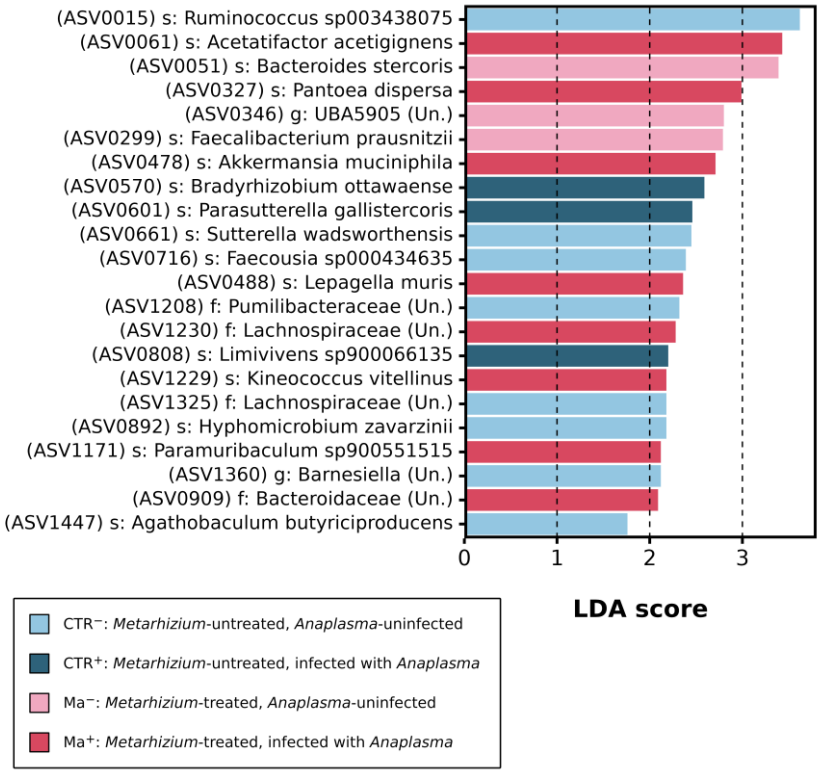

Figure 4. Taxonomic biomarkers identified by LEfSe analysis at the ASV level. The bar graph shows the taxonomic biomarkers identified by Linear Discriminant Analysis Effect Size (LEfSe) analysis, with the best taxonomic classifications obtained for the ASVs considered biomarkers. The colors of the bars indicate the treatment group that is most strongly associated with each biomarker. The displacement along the X-axis represents the Linear Discriminant Analysis (LDA) values. Statistical thresholds: $p < 0.05$ and LDA > 1.5.

3.4. Bacterial co-occurrence network analysis

Co-occurrence networks at the genus level (Table 2, Figure 5) revealed distinct structural patterns across treatments. The CTR- and Ma+ treatments exhibited the highest number of nodes, with 92 and 85 nodes, respectively. Negative edge correlations predominated in the CTR- (57.23%) and Ma+ (55.44%) networks, suggesting competitive interactions, whereas positive edge correlations were more frequent in the CTR+ (59.42%) and Ma- (53.06%) networks, indicating cooperative relationships among species. These findings align with the centrality measures (Table 2), where Ma+ showed the highest average degree of connections (4.541 ± 3.115), exhibiting significant differences, followed by CTR- (3.457 ± 2.201). Additionally, CTR - and Ma+ exhibited the highest average betweenness (0.032 ± 0.046

and 0.027 ± 0.048, respectively). Keystone analysis (Figure 7) identified *Phocaeicola* and *Faecalibacterium* as key genera in CTR-, whereas *Prevotella, Blautia, Gemmiger*, and QALR01 (Oscillospirales) were keystone genera in the Ma+ bacterial community.

The Ma- treatment displayed the most fragmented network structure (Table 2), with 12 clusters and the highest modularity coefficient (0.801). The smaller network diameter (6) reflects a compact, localized structure. Centrality measures revealed that Ma- had the highest average Closeness value (0.606 ± 0.248).

Robustness analysis (Figure 6) revealed that the CTR+ treatment was the most fragile, losing 80% connectivity faster than other treatments under all node removal methods (Betweenness, Degree, Cascading, and Random). In contrast, the Ma+ and Ma- treatments exhibited greater resilience and maintained network connectivity, even with progressive node removal.

Co-occurrence networks at the genus level (Table 2, Figure 5) revealed distinct topological organizations among treatments, directly impacting their responses to Robustness analysis (Figure 6). The CTR- network presented the highest number of nodes (92) and edges, with a predominance of negative correlations (57.23%). This structure was reflected in its centrality metrics, where it exhibited the second highest average Degree (3.457 ± 2.201) and a high average Betweenness (0.032 ± 0.046) (Table 2). Keystone analysis for this group identified *Phocaeicola* and *Faecalibacterium* as key genera (Figure 5). Complementarily, the robustness evaluation (Figure 6) demonstrated that the CTR- network showed a more gradual loss of connectivity under different node removal scenarios compared to CTR+. With the presence of *Anaplasma marginale* (CTR+), although it presented 72 nodes, this network was distinguished by an inversion in interactions, showing a higher proportion of positive edges (59.42%), an average Degree (1.917 ± 1.361), and Betweenness (0.027 ± 0.057) statistically different from CTR- (Table 2). In the robustness analysis, the CTR+ network lost 80% of its connectivity remarkably faster than the other treatments, regardless of the node removal method (Figure 8).

Regarding treatments involving *Metarhizium*, the Ma- group stood out for its most fragmented network structure, with 53 nodes, but organized into 12 clusters and presenting the highest modularity coefficient (0.801) and smallest diameter (6). Centrality metrics confirmed this characteristic, with Ma- recording the highest average Closeness value (0.606 ± 0.248), indicating that nodes were, on average, closer to each other (Table 2). Ma- network exhibited a loss of connectivity progressively, with the network maintaining a substantial level of

connectivity even after the removal of a considerable fraction of its nodes (Figure 6). The Ma+ treatment, with 85 nodes, showed a predominance of negative correlations (55.44%). However, this network differentiated itself by presenting the highest average degree of connections (4.541 ± 3.115) and a comparable average Betweenness (0.027 ± 0.048) (Table 2). Genera such as *Prevotella, Blautia, Gemmiger*, and QALR01 were identified as keystone for this group (Figure 7). The robustness evaluation for the Ma+ network demonstrated a pattern of connectivity loss remarkably similar to that of Ma-, with both *Metarhizium*-treated networks exhibiting a greater capacity to maintain network connectivity under node removal (Figure 6).

Table 2. Topological characteristics and centrality measures of the co-occurrence networks of the genera present in the treatments.

Parameters	CTR-	CTR+	Ma-	Ma+
Number of Nodes	92	72	53	85
Number of Edges	159	69	49	193
Positives Edges	68 (42.77%)	41 (59.42%)	26 (53.06%)	86 (44.56%)
Negative Edges	91 (57.23%)	28 (40.58%)	23 (46.94%)	107 (55.44%)
Number of Clusters	3	9	12	3
Modularity Coefficient	0.0372	0.457	0.8013	0.0702
Diameter	9	12	6	10
Average Degree	3.457 (± 2.201)^{b}	1.917 (± 1.361)^{c}	1.849 (± 1.045)^{c}	4.541 (± 3.115)^{a}
Average Betweenness	0.032 (± 0.046)^{a}	0.027 (± 0.057)^{b}	0.003 (± 0.005)^{c}	0.027 (± 0.048)^{a}
Average Closeness	0.276 (± 0.152)^{c}	0.387 (± 0.302)^{c}	0.606 (± 0.248)^{a}	0.329 (± 0.154)^{b}

Comparison of average values of Degree, Betweenness and Closeness of bacterial co-occurrence networks between treatment groups. Statistical analysis was performed using two-way ANOVA on ranks ($p < 0.05$), with group differences indicated by different letters based on Fisher's LSD post-hoc test. Treatments: (CTR-) *Metarhizium*-untreated, uninfected with *Anaplasma*; (CTR+) *Metarhizium*-untreated, infected with *Anaplasma*; (Ma-) *Metarhizium*-treated (1 × 108 conidia. mL-1), uninfected with *Anaplasma*. (Ma+) *Metarhizium*-treated (1 × 10^{8} conidia. mL^{-1}), infected with *Anaplasma*.

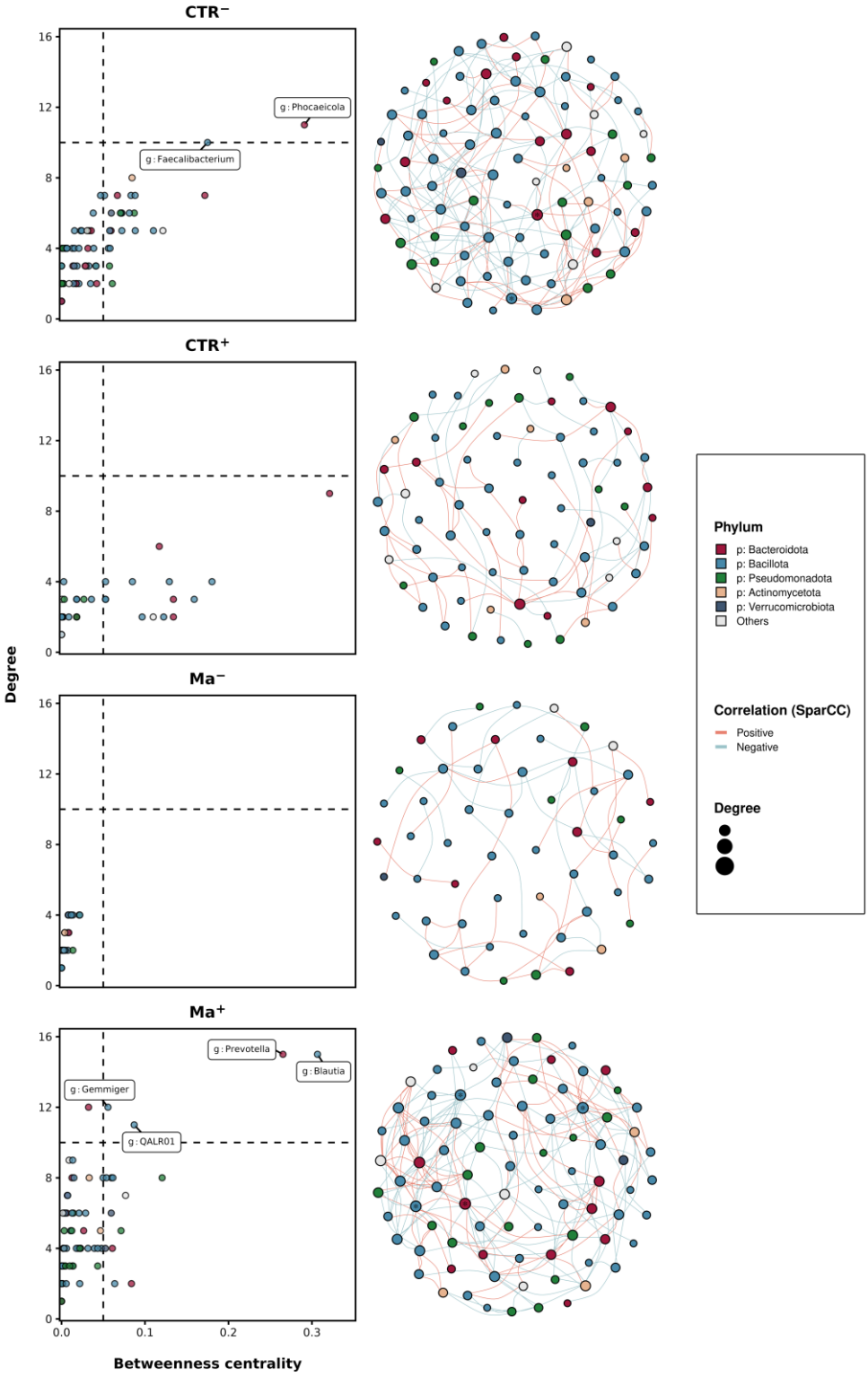

Figure 5. Co-occurrence networks of bacterial genera between the treatments. Co-occurrence networks representing positive (red) and negative (blue) correlations between bacterial genera in each treatment group. Correlations were determined using Sparse Correlations for Compositional Data (SparCC) analysis with statistical thresholds of $p < 0.05$, SparCC > 0.5 for

positive correlations, or < -0.05 for negative correlations. Keystone analysis with degrees of linkages (degree) > 10 and betweenness centrality (betweenness centrality) > 0.05 are marked with asterisks in the network (right) and identified in the dot plot correlating these two parameters (left). Treatments: (CTR-) *Metarhizium*-untreated, uninfected with *Anaplasma*; (CTR+) *Metarhizium*-untreated, infected with *Anaplasma*; (Ma-) *Metarhizium*-treated, uninfected with *Anaplasma*. (Ma+) *Metarhizium*-treated, infected with *Anaplasma*.

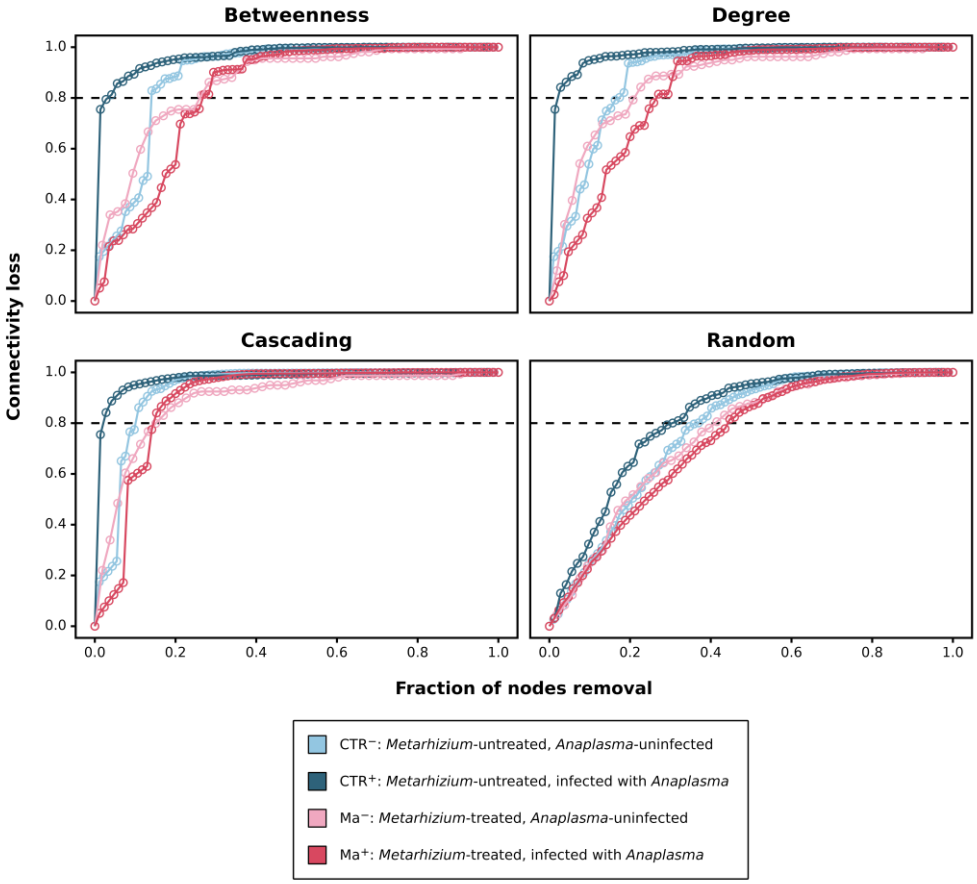

Figure 6 Robustness analysis of bacterial co-occurrence networks between the treatments. Graphs representing the loss of connectivity of the co-occurrence networks of each treatment under different node removal scenarios: targeted attack removing nodes with the highest Betweenness Centrality and linkage degrees (top), cascade effect with sequential removal and recalculation of Betweenness Centrality (bottom left), and random removal of taxa (bottom right).

3.5. Functional composition analysis

The results did not show distinct grouping of KEGG classes among the treatments

(Figure 7A). The most abundant functional classes in relative terms were Carbohydrate Metabolism (13.17%), Amino Acid Metabolism (11.83%), and Cofactor and Vitamin Metabolism (9.63%), reflecting essential metabolic pathways prevalent in all samples. Functional diversity analysis revealed that the CTR treatment had a significantly lower mean diversity than the other treatments (Figure 7B). In the differential abundance analysis of functions (Figure 7C), volcano plots showed that the groups untreated or untreated with *M. anisopliae* infected with *A. marginale* (CTR+ and Ma+) had a higher number of abundant features. In contrast, in treatments without *A. marginale* infection (CTR- vs. Ma-), the presence of *M. anisopliae* (Ma-) led to a considerable increase in the number of more abundant features. However, in treatments with *A. marginale* infection (CTR+ vs. Ma+), an opposite trend was observed, with CTR+ showing more abundant features than Ma+.

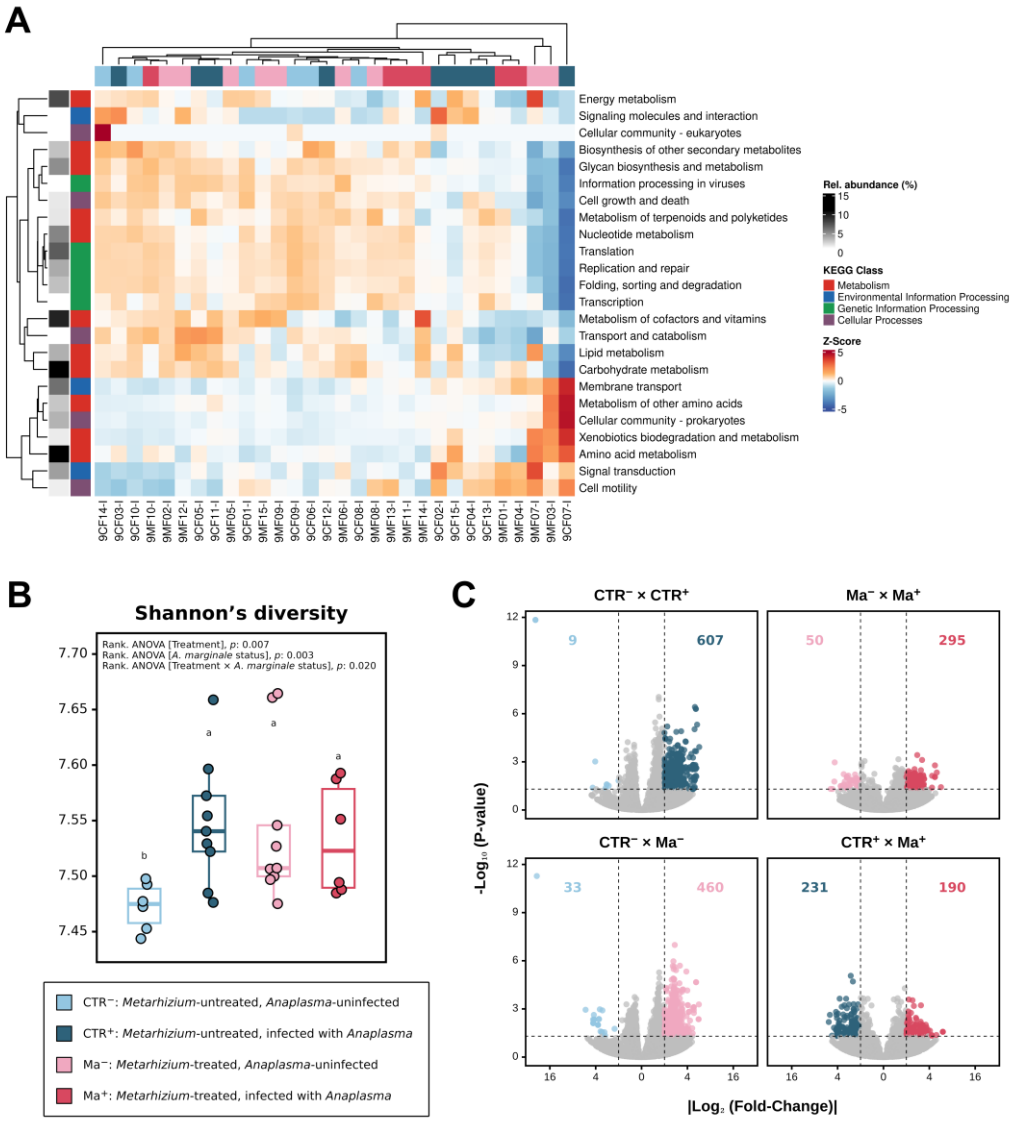

Figure 9. Predicted functional profiles of KEGG orthologs across the treatments. (**A**) Heatmap showing the distribution of KEGG orthologs (KOs) categorized into subclasses of the KEGG classes "Metabolism," "Environmental information processing," "Genetic information processing," and "Cellular processes." Abundances were transformed into Z-scores, with the overall abundance and KEGG class indicated in the left column. Sample treatments are represented by color-coded bars above the heat map. Hierarchical clustering of the rows and columns was performed using the Ward method. (**B**) Boxplots of functional diversity inferred from the Shannon diversity of KOs across treatments. Statistical analysis was conducted using two-way ANOVA on ranks ($p < 0.05$), with group differences indicated by different letters based on Fisher's least significant

4 DISCUSSION

Metarhizium. anisopliae infection can trigger systemic immune responses in ticks (Fiorotti *et al*., 2022), which may interfere with the establishment and persistence of *A. marginale* in the arthropod host. Accordingly, fungal infections may have broader implications beyond direct tick mortality, potentially affecting pathogen transmission dynamics. In Brazil, *A. marginale* has a high prevalence among cattle, leading to substantial economic losses in the livestock industry (Rodrigues *et al*., 2019). Therefore, integrating biological control methods, such as entomopathogenic fungi, with an understanding of their effects on tick-pathogen interactions, could offer a more sustainable and effective approach to managing *R. microplus* populations and reducing the incidence of bovine anaplasmosis.

M. anisopliae isolate LCM S04 was selected because of its known virulence potential against *R. microplus* (Mesquita *et al*., 2020; Correa *et al*., 2021; Borio *et al*., 2022; Mesquita *et al*., 2023) and its ability to reach the tick gut within 72 h, as detected by qPCR in two out of 15 samples from the Ma+ group. However, *M. anisopliae* DNA was not detected by qPCR in any of the gut samples from Ma−. This variability may be related to individual differences in host response to the fungus topical treatment. However, the non-detection of *M. anisopliae* in the gut does not necessarily indicate that cuticular penetration did not occur. Arruda *et al*. (2005) observed, through optical microscopy, extensive fungal penetration on the surface of *R. microplus* 72 hours after treatment with *M. anisopliae*. As the fungus penetrates, the host activates immune response pathways, even if the fungus has not yet reached all tick organs (Fiorotti *et al*., 2019; Fogaça *et al*., 2021). Furthermore, colonization of the hemolymph by *M. anisopliae* may trigger systemic signals that affect distant tissues (including the gut), proposing a potential "off-target" modulation of the tick microbiota by the fungus. "Off-target" modulation refers to the ability of a biological agent to affect a specific organ without directly acting on it. Similar effects have been observed with other biological agents, such as *Bacillus thuringiensis*, which affects both gut cells and the fat body in insects (Bravo *et al*., 2007). Therefore, without direct detection in all samples, *M. anisopliae* may still influence tick microbiota and physiology via indirect systemic mechanisms. Future studies could evaluate multiple time points post-infection to capture dynamic microbiome changes, may be important for temporal shifts in microbial communities.

Tick microbiota is a complex and dynamic system composed of bacteria, viruses, fungi, and protozoa that colonize the gut, salivary glands, ovaries, and other organs. This dynamic can be influenced by intrinsic and extrinsic factors, with interactions between symbionts,

commensals, and pathogens playing a crucial role in disease ecology (Narasimhan *et al*., 2017; Piloto-Sardiñas *et al*., 2024). The microbial community plays a crucial role in tick physiology, influencing nutrition, development, and ability to transmit pathogens (Bonnet *et al*., 2021), as well as vectorial competence (Wu-Chuang *et al*., 2023). Moreover, these interactions exhibit organ-specific patterns, as demonstrated by Pérez *et al*. (2025), whose findings reinforced the hypothesis that *A. marginale* impacts the microbiota of *R. microplus* at an organ-specific level, modulating salivary gland and ovarian communities differently.

The microbiota of vertebrate hosts, such as the bacteria present on their skin, can directly influence the microbial composition of ticks, particularly in the gut, as reported in studies on host skin bacteria in tick (Bonnet *et al*. 2017). Most tick-borne pathogens are secondarily acquired by ticks while feeding on the infected hosts (Maitre *et al*., 2023). In this study, all *R. microplus* females were fed on the same *A. marginale*-infected bovine, ensuring that they were exposed to a similar microbial and pathogen environment. However, despite this shared feeding source, subsequent qPCR analysis revealed that the ticks exhibited different *A. marginale* loads and infection status, allowing for comparative analysis between groups. This variability underscores the complexity of pathogen acquisition and persistence in *R. microplus*, suggesting that intrinsic factors related to the tick may influence infection outcomes. By implementing a structured experimental design in which ticks were either treated or untreated with *M. anisopliae* and later classified based on their *A. marginale* status, this study provided a controlled framework to assess the interplay between fungal infection and pathogen dynamics.

The use of qPCR with a probe with a specific probe targeting the *msp* 1β gene proved to be an essential tool for the sensitive and specific detection of *A. marginale*, ensuring accuracy in group classification and reinforcing the reliability of the findings (Carelli *et al*., 2007). In their 2021 review, O'Neal *et al*. (2021) listed the points of vector biology that need to be elucidated: survival benefits associated with infection, metabolic modifications during infection, molecular mechanisms of tick immune defense, and interactions with endosymbionts and the microbiome. The present study investigated possible modifications in the bacteriome of *R. microplus* females treated or not with the entomopathogenic fungus *M. anisopliae*, in the presence or absence of *A. marginale* infection. Regarding the results obtained, the macromorphological patterns remained conserved across all treatment groups (Figure 2), this observation may help explain the widely shared bacterial composition between groups, with only a small proportion of exclusive ASVs per treatment. This finding aligns with previous studies on *Ixodes scapularis*, which reported similar patterns between control and *Anaplasma*

phagocytophilum-infected ticks (Cabezas-Cruz *et al*., 2017; Paulino *et al*., 2023)

Regardless of treatment, the taxonomic composition showed a widely shared bacterial composition at the phylum and genus levels (Figure 3). *Bacteroidota* and *Bacillota* were the most dominant phyla observed. At the genus level, *Phocaeicola*, *Prevotella*, and *Bacteroides* were the most abundant taxa. More than half of the taxa (53.4%) were shared between the groups, whereas unique genera were detected at a low frequency (≤3%), with no evidence of specific clustering by treatment.

Differential abundance analysis using LefSE statistical analysis (Figure 4) identified significant taxonomic biomarkers corresponding to 3.15% of the total analyzed ASVs. Among them, CTR-and Ma+ had the highest associated taxa (eight each), while CTR+ and Ma- exhibited only three biomarkers each. All groups presented different predominant taxa. Similarly, Pérez *et al*. (2025) observed that ovaries and salivary glands of *R. microplus* not infected with A. marginale were dominated by *Sphingomonadaceae*, whereas *Nocardiaceae* predominated in infected tissues. In the CTR-, we observed that the basal state of the gut bacteriome of fed ticks, *Ruminococcus*, is the most predominant taxon. In the presence of the pathogen, *Bradyrhizobium ottawaense* was more abundant, indicating that each organ may harbor a distinct bacteriome that changes in response to the presence of A. marginale. This same group, exhibited taxa that are lost under stress, including *Lachnospiraceae* and *Ruminococcus,* which are associated with gut homeostasis in mammals (Khalil *et al*., 2022) and may be transferred from vertebrates to ticks (Maldonado-Ruiz, 2024). Although the basal bacteriome revealed only biomarkers classified as environmental bacteria, this does not imply the absence of natural endosymbionts, but rather that environmental taxa, more variable and adaptable, became statistically more predominant.

In contrast, the individual-factor stress groups, CTR+ (*Anaplasma* infection) and Ma– (*Metarhizium* treatment), exhibited reduced associated taxa. These intermediate conditions produced relatively simplified profiles, with few unique biomarkers. Previous research on *Apis mellifera* (Hymenoptera: Apidae) also revealed that isolated infections by *Nosema ceranae* may have induced lower associated taxa (Sbaghdi *et al*., 2024). On the other hand, Ma+ was simultaneously exposed to *Metarhizium* and *Anaplasma* infection, possibly creating unique ecological conditions with the presence of taxa adapted to challenging environments that appear to reshape the bacteriome, such as *Akkermansia muciniphila*, *Paramuribaculum*, and *Pantoea dispersa*. Although *A. muciniphila* is associated with the gastrointestinal tract of mammals (Cani; Vos, 2017; Yang; Yi; Xia, 2023), *P. dispersa* is an environmental bacterium that can

colonize bovine skin, particularly in environments with high exposure to plants and soil (Yi; Xia, 2023). Several studies suggest that environmental or transient bacteria, even without establishing stable symbiotic relationships, may influence essential physiological functions in the host, such as immune response, metabolic modulation, and pathogen defense (Bonnet *et al*., 2017; Maldonado-Ruiz, 2024). Therefore, the predominant presence of environmental bacteria in all analyzed groups reinforces the idea that microbiota, even when dominated by environmental organisms, can play relevant functional roles in tick microbial ecology. A similar pattern was observed in another arthropod, such as *B. bassiana*-treatment, *Aedes aegypti* (Diptera: Culicidae) larvae and adults, where an increase in environmental bacteria occurred at the expense of the resident microbiota (Yin *et al*., 2024).

Analysis of the co-occurrence networks (Figure 5) exhibited significant differences between the treatments, highlighting the network from the guts of ticks treated with fungus and negative *for A. marginale* (Ma-). This group exhibited highly modular structure (12 clusters, modularity = 0.801) with a shorter average distance between nodes (Closeness = 0.606) (Table 2). This suggests that *M. anisopliae* may have induced a disturbance in the natural microbial interactions within the tick gut. This compartmentalization may reflect a breakdown of the ecological relationships in untreated and uninfected tick bacteriome potentially compromising host physiological stability and favoring the effectiveness of biological control strategies. Previous studies have shown that changes in network structure, especially those involving the removal of keystone taxa, can impair host functions, as observed in ticks with dysbiosis (Wu-Chuang *et al*., 2023). This destabilization may benefit biological control, as altered networks reduce the tick's resilience to stressors (Mateos-Hernández *et al*., 2020). The Ma- robustness analysis showed network stability despite node removal, which may result from reduced dependence on central taxa, thereby artificially enhancing resistance to disturbance.

Despite the differences in topo analyses and centrality measures (Table 2), the group treated and infected with *A. marginale* exhibited a similar network outcome, maintaining connectivity under perturbation (Figure 5). Although Pérez *et al*. (2025) reported that *A. marginale* significantly compromises the robustness of bacterial networks in salivary and ovarian glands, our results exhibit fungal-induced microbiota disruption, in combination with *A. marginale* infection, led to a reorganization of the microbial community, with a dominance of negative interactions (55.44%). Although the robustness pattern resembled that observed in the Ma- group, the ecological nature and functional implications of these interactions are likely distinct. These results support the hypothesis that microbial disturbance, whether induced by

the fungus alone or through its interaction with the pathogen, may alter community dynamics, creating a less favorable environment for host stability (Mateos-Hernández *et al*., 2020).

Additionally, when comparing the results of the co-occurrence networks with those of Paulino *et al*. (2023), groups of *Ixodes ricinus* without bacterial infection exhibited greater diversity and predominance of negative interactions. In contrast, groups infected with *A. phagocytophilum* exhibited a higher proportion of positive links, suggesting that more cohesive networks adapted to the conditions imposed by infection. In addition, infected networks without fungal treatment presented a higher vulnerability to changes in node removal from the network. These similarities highlight the common patterns of microbial reorganization in response to the presence of pathogens.

Functional prediction analysis (Figure 7) allowed us to estimate the potential of microbial communities based on taxonomic composition, which in this study did not identify a distinct functional grouping among treatments, indicating relative stability in metabolic functions. The main classes observed were Carbohydrate Metabolism 13,17%, Amino Acids 11,83% and Cofactors/Vitamins 9,63%, which were essential in all samples. The prominence of amino acid and vitamin metabolism is particularly relevant given that *Anaplasma marginale*, similar to other *Rickettsiales*, presents a reduced genome and lacks complete pathways for amino acid biosynthesis, relying instead on the host or cohabiting microorganisms to supply these metabolites (Brayton *et al*., 2005; Dunning Hotopp *et al*., 2006). Genomic analyses have shown that *A. marginale* retains only the terminal enzymes for the synthesis of eight amino acids, while maintaining intact pathways for nucleotides and lipids, underscoring its dependence on external amino acid sources (Brayton *et al*., 2005). Therefore, the enrichment of these functional categories may reflect microbial adjustments that provide critical nutrients, sustaining both the host and the intracellular pathogen. In terms of functional diversity, CTR- exhibited lower functional diversity, suggesting that the present of *A. marginale* and *M. anisopliae* may increase functional diversity. This lower functional diversity in the absence of stressors may reflect not only a less activated immune response, but also the lack of microbial manipulation typically induced by pathogens. In the presence of *A. marginale* or *M. anisopliae*, increased functional diversity may arise from the microbial community's adaptation to support host or pathogen survival, potentially by enhancing nutrient availability and metabolic plasticity (Hajdušek *et al*., 2013; Fogaça *et al*., 2021).

This agrees with the increased abundance of predicted functional traits (KEGG orthologs) observed in the untreated-infected and treated-infected groups, indicating that *A.*

marginale infection may be associated with an increase in the abundance of specific functional genes, suggesting a higher expression or prevalence of these functions. The activation of immune pathways in response to infection or fungal treatment may drive these functional changes, as observed in insect systems with stress-induced immune responses (Myllymäki *et al*., 2014). In treatments without *A. marginale* (untreated and treated), the *M. anisopliae*-uninfected group was associated with a greater abundance of functional genes, indicating a modulatory effect of the fungus on the microbiota. In contrast, in *A. marginale* (CTR+ versus Ma+), the untreated-infected group presented more abundant traits, suggesting that infection reduced the modulatory effect of the fungus. This could be due to the competitive interactions between the immune responses elicited by the pathogen and fungus, highlighting the complex interplay between stress, immune activation, and microbiota modulation (Belkaid; Hand, 2014; Levy *et al*., 2017).

Microbiome analysis revealed that although the overall bacteriome composition remained stable, treatment with *M. anisopliae* supported a microbial community with more efficient and stabily ecological interactions, even in the presence of *A. marginale*-infected ticks. Biomarker analysis (LEfSe) indicated that the absence of *Metarhizium* and *Anaplasma* (CTR-) or the combination of treatment and infection (Ma+) favored a more complex microbiota, while infection with *A. marginale* increased the abundance of specific functional genes. The combination of fungus and pathogen appears to strengthen the stability of the microbiota without compromising the action of the fungus. This study pioneered the exploration of the gut bacteriome modulation in *R. microplus* treated with *M. anisopliae* in the presence of *A. marginale*, providing valuable insights into integrated control strategies for ticks and vector-borne diseases. These results highlight the potential of using entomopathogenic fungi as a sustainable approach to tick management, with significant implications for animal and livestock health.

5 CONCLUSION

These findings suggest that:

- Ticks simultaneously exposed to *Metarhizium* and *Anaplasma* exhibited a reshaped bacteriome, characterized by a greater number of associated taxa and a distinct microbial composition compared to the control and individually infected groups.
- The highly connected Ma+ network and robustness may reflect a compensatory response of the microorganism and host immune system to dual infection, rather than a

sign of bacteriome stability or benefit.

- Functional prediction based on ASV phylogenetic placement indicated fungus and bacteria associate may arise from the bacteria community's adaptation to support host or pathogen survival.
- This is supported by the increased abundance of predicted functional genes (KEGG orthologs) (Ma+) bacteriome may respond to infection by shifting its metabolic functions to adapt to the altered physiological environment.

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CHAPTER II

IMMUNE RESPONSE OF *Noctua pronuba* LARVAE TO ENTOMOPATHOGENIC AND NON-ENTOMOPATHOGENIC FUNGI

ABSTRACT

Noctua pronuba is an agriculturally important pest, widely distributed and resilient to low temperatures. Despite its ecological and economic impact, little is known about its immune responses to fungal agents. This study aimed to investigate and compare the immune responses of *N. pronuba* larvae exposed to the entomopathogenic fungus *Beauveria bassiana* and the non-entomopathogenic fungus *Trichoderma atroviride*, evaluating how the insect immune system discriminates between these two fungal challenges. Fourth-instar larvae were immersed in conidial suspensions (4×10^8 conidia/mL) of each fungus. Gene expression of *Cecropin*, *Attacin*, and *Peptidoglycan recognition protein A* (*PGRP-A*) was evalueted by qPCR at 6-, 12-, and 24-hours post-treatment (hpt), allowing temporal evaluation of the immune response. At 6 hpt, exposure to *B. bassiana* induced immune activation, with significant upregulation of *Attacin* and *PGRP-A* ($p < 0.05$), indicating recognition of the fungus as a pathogenic threat. In contrast, *T. atroviride* elicited weaker modulation, with gene expression close to baseline or downregulated compared to controls. At 12 hpt, expression of all three genes was suppressed, with *PGRP-A* showing the lowest levels in larvae treated with *T. atroviride* ($p < 0.05$), suggesting an entomopathogenic effect. At 24 hpt, both fungi-maintained suppression of *Attacin* and *PGRP-A* ($p < 0.0001$), suggests that the fungi modulate the host's humoral immune response, potentially affecting early defense mechanisms. These results demonstrate that the same immune genes may respond differently depending on the fungal species, and that both *B. bassiana* and *T. atroviride* can modulate humoral immune responses in *N. pronuba*. This study provides the first detailed characterization of antifungal immunity in *N. pronuba*. The findings expand knowledge of insect–fungus interactions and support the development of targeted and environmentally sustainable pest management strategies. Future research integrating immune responses, microbiota composition, and fungal infection dynamics will be essential to strengthen biological control approaches against *N. pronuba*.

Keywords: Innate immune response; Biocontrol fungi; Gene expression; Lepidopteran pests; Integrated pest management

1 INTRODUCTION

Noctua pronuba (Linnaeus) (Lepidoptera: Noctuidae), commonly known as the "large yellow underwing" or "winter cutworm," is native to Western Asia, North Africa, and Europe, and is recognized for its migratory ability and resistance to low temperatures, tolerating temperatures of 4 °C or below (Green *et al*., 2016; Chapman *et al*., 2010). This species has been introduced and spread in regions of North America, including the United States and Canada, where it represents a significant threat to various agricultural crops (Passoa; Hollingsworth, 1996; DiFonzo; Russell, 2010; Morris, 1987; Copley; Cannings, 2005).

The larvae of *N. pronuba* cause direct economic losses by feeding on the leaves of a wide variety of crops, including alfalfa, oats, rye, wheat, vegetables (carrot, onion, potato, spinach, rhubarb, beet, tomato), lawns, hay, small fruits (strawberries and grapes), ornamentals (carnations and chrysanthemums), and weeds (Green *et al*., 2016). Climate change is expanding the geographic distribution and host range of *N. pronuba*, emphasizing the growing need for sustainable and effective management strategies (Tărău *et al*., 2023).

The control of this cutworm relies on chemical insecticides (pyrethroids, chlorpyrifos, and carbaryl), to which early larval stages are more susceptible than mature stages (Difonzo; Russell, 2010; Green *et al*., 2016). However, sustainable agricultural practices require alternatives that minimize environmental impact, such as entomopathogenic microorganisms. Entomopathogenic fungi are capable of infecting and causing disease in arthropods, representing key ecological agents for biological control (Lacey *et al*., 2001). Among the most studied and applied genera in agriculture are *Metarhizium*, *Beauveria*, *Isaria*, *Cordyceps*, *Paecilomyces*, and *Lecanicillium*, widely used due to their effectiveness and safety (Mascarin; Jaronski, 2016; Litwin *et al*., 2020).

The entomopatogenic fungi *B. bassiana* is a successful biocontrol agent, naturally occurring in soils and capable of endophytic colonization, and is used as a mycoinsecticide against various pests, including Lepidoptera, Coleoptera, and Diptera (Toledo *et al*., 2014; Maistrou *et al*., 2018; Shahriari *et al*., 2021). In contrast, the filamentous non-entomopathogenic fungus *Trichoderma atroviride* is widely used as a biofungicide in agriculture due to its ability to parasitize pathogenic fungi through mycoparasitism (Kubicek *et al*., 2019). Recent studies indicate that *Trichoderma* species may also directly affect insect pests through the production of insecticidal, antifeedant, and repellent metabolites (Poveda, 2021).

To infect the arthropod host, these fungi develop specialized structures, such as germ tubes and penetration hyphae, which confront the first barrier of defense, the cuticle, composed

of hydrocarbons and chitin, whose degradation is facilitated by fungal enzymes (Hajek; St. Leger, 1994; Ortiz-Urquiza; Keyhani, 2013). Once this barrier is breached, fungi face the insect immune system, which acts via cellular responses (phagocytosis, nodulation, encapsulation, melanization) mediated by hemocytes, and humoral responses, including antimicrobial peptide (AMP) synthesis, the prophenoloxidase cascade, and reactive oxygen and nitrogen species production (Lavine; Strand, 2002; Cerenius *et al*., 2008; Tsakas; Marmaras, 2010).

Pattern recognition receptors (PRRs) play a crucial role in detecting invading microorganisms, recognizing components such as fungal β-1,3-glucans, bacterial peptidoglycans, and lipopolysaccharides, activating immune signaling pathways such as Toll and Imd, which regulate the expression of AMPs including *Attacins*, *Cecropins*, and *Defensins* (Gillespie *et al*., 2000; Wang *et al*., 2010; Shahriari *et al*., 2021; Yao *et al*., 2023). These specific responses allow the insect to modulate defenses against different types of fungi.

Although *Trichoderma* is a genus of filamentous fungus, unlike *B. bassiana*, which penetrates the insect cuticle and causes mortality, *Trichoderma* spp. are widely recognized for their role in biological control through the production of bioactive molecules with antagonistic effects on plant-pathogenic fungi and nematodes (Druzhinina *et al*., 2018; Zhu *et al*., 2022). These compounds, including secondary metabolites and cell wall-degrading enzymes, enhance crop resistance, reduce the incidence of plant diseases, and promote plant growth (Kubicek *et al*., 2019; Yao *et al*., 2023). *Trichoderma* species are saprophytic fungi with rapid mycelial growth and high environmental adaptability, which allows them to colonize plant roots, outcompete pathogens for space and nutrients, and directly inhibit pathogen development (Yao *et al*., 2023). Although not considered a classical entomopathogenic fungus like *B. bassiana*, *Trichoderma* spp. has been reported to exert direct effects on insects through parasitism and the production of insecticidal secondary metabolites, antifeedant compounds, and repellents (Poveda *et al*., 2021).

In lepidopteran species closely related to *N. pronuba*, such as *Spodoptera frugiperda*, entomopathogenic fungi have been shown to suppress cellular immunity, reducing hemocyte counts and nodulation, while simultaneously activating humoral responses, including antimicrobial peptide production (Wang, 2022). These findings provide a comparative framework for interpreting immune responses in *N. pronuba* exposed to fungal pathogens. Understanding interactions between lepidopteran larvae and fungi extends beyond pathogenicity. Even non-pathogenic fungi present in the larval environment can modulate immune readiness and influence host susceptibility to pathogens (Rahimi *et al*., 2019; Shahriari

et al., 2021). Recognizing this complexity underscores the ecological significance of fungal exposure and supports the development of integrated biocontrol strategies that leverage natural immune modulation.

Despite the agricultural and ecological importance of *N. pronuba*, scientific information on the species-specific immune aspects remains limited. This study constitutes the first detailed investigation of key immune gene expression in *N. pronuba* larvae exposed to these fungi, filling an important gap in entomological knowledge and providing data to support the development of more effective and sustainable biological control strategies.

This study aims to investigate immune responses of *N. pronuba* larvae to entomopathogenic fungi, such as *B. bassiana*, and non-entomopathogenic fungi, such as *Trichoderma* spp., commonly present in the larval environment. By comparatively analyzing the expression of genes associated with chemical and immune defenses, the study seeks to elucidate molecular mechanisms underlying fungal pathogenicity selectivity and contribute to the development of safer and more efficient biological control strategies in integrated pest management.

2 MATERIAL AND METHODS

2.1 Study site description

The experimental studies and gene expression analyses using real-time PCR were conducted at the Institute for Sustainable Horticulture, Kwantlen Polytechnic University (KPU), Langley, British Columbia, Canada.

2.2 Experimental design

The study was designed in four main stages (Figure 1), aiming to investigate the immune response of the *Noctua pronuba* larvae when exposed to different fungi:

- Insect exposure to fungi: *N. pronuba* larvae were exposed via bioassay to the fungi *Beauveria bassiana* and *Trichoderma atroviride*. Larvae tissue (whole body) samples were collected at different time points post-exposure (6, 12, 24hours) for subsequent analysis.
- Total RNA extraction: Following collections, total RNA was individually extracted from each moth tissue sample, ensuring integrity and quality for the molecular analyses that followed.
- cDNA Synthesis and qPCR amplification: The extracted total RNA was then reverse-transcribed into cDNA. From this, quantitative real-time PCR (RT-qPCR) was performed to evaluate the expression of specific genes related to *N. pronuba's* innate immunity, as well as a reference gene for normalization.
- Gene expression analysis and biological interpretation: The gene expression data obtained by qPCR were subjected to rigorous statistical analyses to determine significant differences between treated and control groups, allowing for the identification of immune response modulation (activation or suppression) in the insects when facing each fungus.

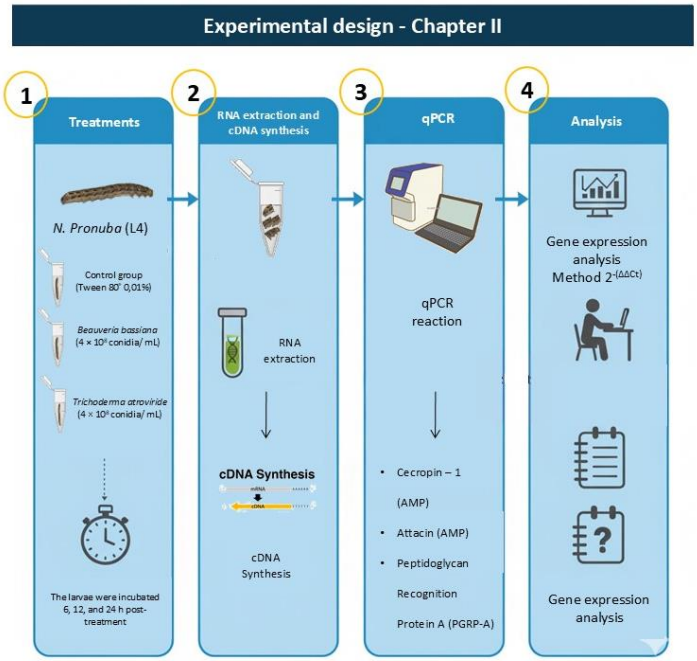

Figure 1: Experimental methodology design for Chapter 2. The scheme illustrates the four main stages of the study on the immune response of *Noctua pronuba* to exposure to entomopathogenic and non-entomopathogenic fungi.

2.3 Source and maintenance of *Noctua pronuba* larvae

N. pronuba larvae were obtained from Eco-Care Technologies Inc. (Langley, BC, CA). The larvae were maintained in the laboratory at 17 ± 1 °C, 75 ± 5% relative humidity (R.H.), under light and dank conditions at 12:12 h under aseptic conditions. The feeding was done with McNeil's full regular diet: 65 g agar powdered, 12.8 g ascorbic acid, 128g brewer's yeast, 16 g methylparaben, 852 g pinto beans dried and ground, 4 g sorbic acid, 16 g vanderzant vitamin mix, 1440 ml water for agar and 3200 ml for dried ingredients.

2.4 Fungal suspension

Beauveria bassiana (CC 372) and *Trichoderma atroviride* (CC 222) were grown on potato dextrose agar (PDA) under controlled conditions (24 ± 1 °C; RH ≥ 80%). After 14 days of incubation, a suspension of conidia containing 4×10^8 conidia/ml was prepared in 0.01 % (v/v) polyoxymethylene sorbitan monooleate-Tween 80® (Fisher Bioreagents) using a hemocytometer. Conidial viability was determined using the spore viability count method (Grace; Jaronski, 2005). Briefly, samples of the fungal suspension were streaked onto PDA plates with sterile cotton swabs and incubated at 25 °C in the dark. Germination assessments

were performed after 22 hours for *B. bassiana* and 17 hours for *T. atroviride*.

2.5 *Noctua pronuba* larvae treatment

The fourth instar larvae of *N. pronuba* were randomly chosen and divided into three groups with three larvae each: C (control/ larvae immersion in Tween 80® (0.01%)), Bb (larval immersion in *B. bassiana* suspension 4×10^8 conidia ml^{-1}) and Ta (larval immersion in *T. atroviride* suspension 4×10^8 conidia ml^{-1}). After of immersion for 20 s, the larvae were incubated 6, 12, and 24 h post-treatment (hpt) at 24 ± 1 °C; RH ≥ 80%. Due to their size, the larvae were divided into four pieces to ensure adequate penetration of RNAlater® (SIGMA-Aldrich) and then stored in 500 µL of the solution at −80 °C until RNA extraction. In addition, a group of untreated larvae was maintained under the same controlled conditions (17 ± 1 °C, 75 ± 5%) to follow part of the life cycle and to assess the possible influence of external factors on larval survival.

2.6 Expressions of the genes involved in immune responses

2.6.1 RNA extraction and cDNA synthesis

Total RNA was extracted from the whole bodies of individual larvae from each treatment group using the RNeasy Mini Kit® (Qiagen) according to the manufacturer's protocol. At the end of the procedure, the RNA concentration and purity were measure with a spectrophotometer NanoDrop-1000® (Thermo Fisher Scientific) and RNA quality was verified with Agarose gel 1,5%. The RNA was reverse transcribed to cDNA using a qPCR BIO cDNA Synthesis Kit® (PCR Biosystems), following the manufacturer's instructions.

2.6.2 Gene expression assays

The genes selected for this study, *Cecropin 1*, *Attacin,* and *Peptidoglycan recognition protein A* (*PGRP-A*), were chosen because of the central role they play in the immune defense of lepidopterans. *β-Actin* was used as a reference gene (Wang *et al*., 2010) (Table 1). Primer's efficiency was assessed by qPCR using serial dilutions of pooled cDNA (10^{-1} to 10^{-4}). The three technical replicates were considered using a 13 µL of volume containing 5 µL of SYBR® Premix Ex, 2 µL nuclease-free water, 2 µL of each primer (10mM), 2 µL of cDNA template using the Bio-Rad real-time thermal cycler. Thermal cycling conditions were: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, annealing at 62°C for

50 s, 72°C for 2 s, 82°C for 2 s, and 72°C for 10 min, followed by melt curve analysis.

Table 1: Specific primers and reference gene for cDNA amplification

Gene	Forward (5' – 3')	Reverse (5' – 3')	Eficiency (%)
Cecropin1	GTTTGGTAGCAGCGTGCAG	GCTTCACCGAGGACTGCTAT	97,75%
Attacin	GAGTGGGAGCTTCATTAGGG	CGAGGAGCGTTAAAGTCCAG	98.15
PGRP A	CCAGATGTGCTGAGATCGTG	TTTGCCGTTACCACCTACAA	97,35%
β-Actin	CCTGGTATTGCTGACCGTATGC	CTGTTGGAAGGTGGAGAGGGAA	99,6%

Primers from Wang *et al*., 2010.

2.6.3 Calculation of the relative gene expression using a $2^{-(\Delta\Delta Ct)}$ method

Relative gene expression was calculated by the $2^{-(\Delta\Delta Ct)}$ method as described by Livak and Schmittgen (2001). Ct values from technical replicates were used to calculate ΔCt. The ΔCT for each sample was determined by normalizing the Ct of the target gene (*Cecropin 1*, *Attacin,* or *PGRP-A*) to the Ct of the reference gene (*β-Actin*). Finally, the $2^{-(\Delta\Delta Ct)}$ was calculated by normalizing the ΔCt of each treatment group to ΔCt of the control group.

2.7 Statistical analysis

The statistical significance of the differential gene expression (fold change) was assessed by comparing the ΔCT values of the biological replicates for each gene across the *B. bassiana* and *T. atroviride* treatment groups. Normality was checked using the Shapiro-Wilk's test. Parametric data were analyzed by one-way ANOVA followed by Tukey's post-hoc test for pairwise comparisons ($p \leq 0.05$). Non-parametric data were analyzed using the Kruskal-Walli's test followed by Dunn's multiple comparisons test ($p \leq 0.05$).

3 RESULTS

3.1. Expression of immune genes in response to fungal challenge

3.1.1 Six-Hour Post-Challenge

The expression of genes *Cecropin-1*, *Attacin*, and *Peptidoglycan Recognition Protein A* (*PGRP-A*) in *N. pronuba* larvae was analyzed six hours after treatment with *B. bassiana* (Bb) and *T. atroviride* (Ta) (Figure 2). For the *Cecropin-1* gene, expression in *B. bassiana*-treated larvae was 2.80 times higher compared to the control group (CTR). In contrast, the group treated with *T. atroviride* showed a 1.32 decrease in expression; no statistically significant difference was observed between them (p= 0.1771). The *Attacin* gene was 7.23 times more highly expressed in the Bb group, and 1.11 times more expressed in the Ta group relative to the CTR group. There was a statistically significant difference between the Bb and Ta groups (p= 0.026). Lastly, *PGRP-A* expression was significantly different when comparing the Bb group (8.25 times higher expression) and the Ta group (1.11 times lower expression) relative to the CTR (p= 0.04).

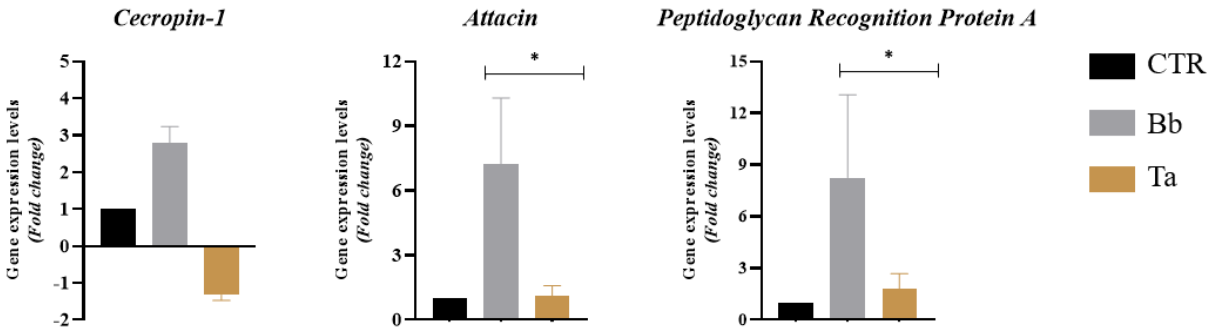

Figure 2. Gene expression levels (Fold change) (± SEM) in *Noctua pronuba* larvae in response to fungal treatment with *Beauveria bassiana* (CC 372) and *Trichoderma atroviride* (CC 222) 6 hours after treatment. Gene expression was demonstrated relative to *β-Actin* as the reference gene. Asterisks indicate statistical difference by the Kruskal-Walli's test followed by Dunn's test ($p < 0.05$).

3.1.2 Twelve-Hour Post-Challenge

In contrast to the previously assessed time point, twelve hours after treatment (Figure 3), the expression of the analyzed genes was significantly different. The expression of the

Cecropin-1 gene was 5.94 times lower in larvae from the Bb group and 6.61 times lower with Ta. Both treatments showed a statistically significant difference relative to the control group ($p \leq 0.0001$). Similarly, *Attacin* was 6.08 times less expressed in the presence of Bb and 5.00 times less expressed in the presence of Ta, both showing a statistically significant difference compared to the CTR group ($p < 0.0001$). The expression of *PGRP-A* was 5.80 times less expressed in Bb and 10.19 times less expressed in Ta. Both reductions were statistically significant relative to the control ($p < 0.0001$). Additionally, a statistically significant difference was observed between the *B. bassiana* and *T. atroviride* groups ($p= 0.0274$).

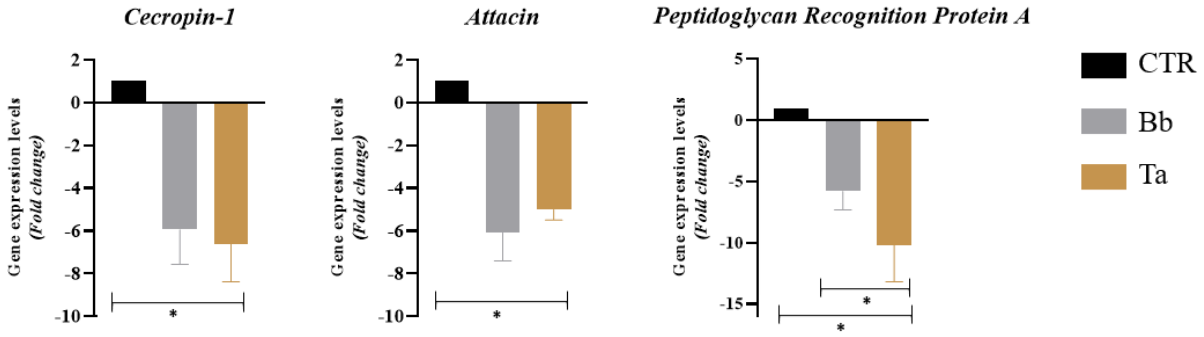

Figure 3: Gene expression levels (Fold change) (± SEM) of *Noctua pronuba* larvae in response to fungal treatment with *Beauveria bassiana* (CC 372) and *Trichoderma atroviride* (CC 222) 12 hours after treatment. Gene expression was demonstrated relative to *β-Actin* as the reference gene. Asterisks indicate statistical difference by the one-way ANOVA test followed by Tukey's test ($p < 0.0001$).

3.1.3 Twenty-four-Hour Post-Challenge

Regarding the last analyzed time point (Figure 4), after 24 hours of treatment, *Cecropin-1* expression was 1.57 times higher in the Bb group and 2.64 times lower in the Ta group, both compared to the CTR group. However, these differences were not statistically significant ($p= 0.1767$). The *Attacin* gene was 7.55 times less expressed in Bb and 3.90 times less expressed in Ta. The reduction in expression was statistically significant relative to the CTR ($p = 0.0038$). The *PRPG-A* gene was 1.03 times less expressed in Bb and 3.85 times less expressed in Ta. Only the reduction observed in the Ta group was statistically significant relative to the control ($p < 0.0001$). Additionally, a statistically significant difference was observed between the *B. bassiana* and *T. atroviride* groups ($p= 0.0093$).

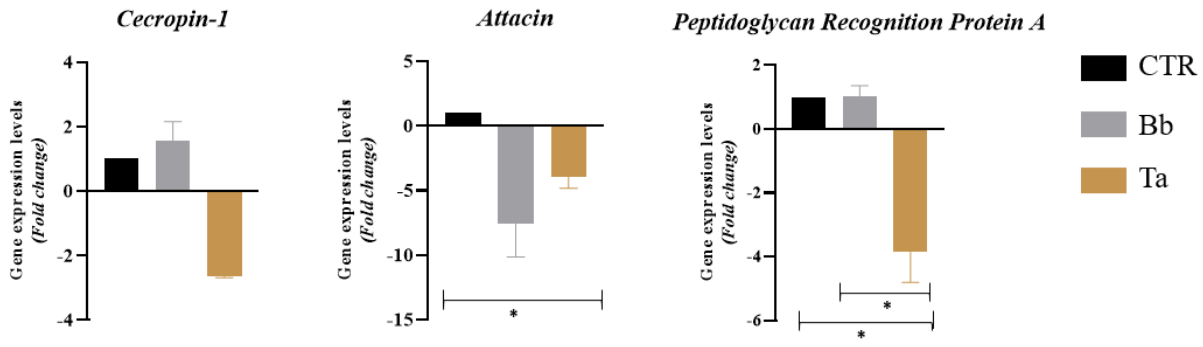

Figure 4: Gene expression levels (Fold change) (± SEM) of *Noctua pronuba* larvae in response to fungal treatment with *Beauveria bassiana* (CC 372) and *Trichoderma atroviride* (CC 222) 24 hours after treatment. Gene expression was demonstrated relative to *β-Actin* as the reference gene. Asterisks indicate statistical difference by the Kruskal-Walli's test followed by Dunn's test ($p \leq 0.0001$).

4 DISCUSSION

Chemical insecticides are the main form of management for controlling insect pests in major agricultural production systems. The intensive and sometimes inadequate application of these products generates environmental and agricultural impacts (Le Goff; Giraudo, 2019). In parallel, the effects of climate change, the increase in the world population, and the concern for food security are increasing interest in sustainable agricultural practices, including bioproducts for pest control (Skendžić *et al*., 2021).

The use of fungi as biocontrol agents stands out for the wide variety of biotechnological applications such as biofertilizers, mycorrhizae, insect and nematode control, endophytic fungi, weed biocontrol, post-harvest disease control, and production of plant growth-promoting hormones (Hyde *et al*., 2019). Their use can be integrated into Integrated Pest Management (IPM) to combine biological strategies with predators, parasitoids, and microorganisms to reduce environmental impact and enhance control (Vázquez, 2019). The most studied and widely used genera in agriculture are *Metarhizium*, *Beauveria*, *Isaria*, *Ophiocordyceps*, *Cordyceps*, *Torubiella*, *Pochonia*, *Hirsutella*, *Paecilomyces*, and *Lecanicillium* (Litwin *et al*., 2020), with *B. bassiana* being the entomopathogenic fungus widely studied for the control of lepidopterans.

The effectiveness of *B. Bassiana* against Lepidoptera of the family Noctuidae is reported by Boulamtat *et al*. (2025), where through direct spraying (1×10^8 conidia/mL) on *H. armigera* larvae, they achieved 100% mortality after 12 days of treatment. In *Spodoptera littoralis*, Fergani and Refaei (2021) demonstrated that the indigenous isolate Y-F_ITS1 of *B. bassiana* was highly effective against different developmental stages, with second-instar larvae being most susceptible, showing corrected mortality rates ranging from 85.0 to 99.0% at a concentration of 1×10^9 spores/mL. The study also showed an ovicidal effect, reaching 100% mortality of eggs at the highest concentrations (1×10^8 and 1×10^9 spores/mL), in addition to a complete reduction in adult emergence when pupae were treated with these same concentrations. These results reinforce the fungus's ability to infect and cause high mortality in Noctuidae species, validating its use as a biocontrol agent.

Trichoderma spp. as a biocontrol agent against pathogens is better known as mycoparasitism, attacking and degrading the cell walls of other phytopathogenic fungi (Moreno-Ruiz *et al*., 2020; Rajani *et al*., 2021). The different responses to interaction with other organisms depend on the secondary metabolites produced by different species/strains of *Trichoderma*, which have different levels of toxicity (Moreno-Ruiz *et al*., 2020; Mukherjee *et*

al., 2012; Oros; Naár, 2017; Alizadeh *et al*., 2024). Although little studied, some works suggest that *Trichoderma* has an entomopathogenic potential. *Trichoderma hamatum* demonstrated effectiveness against *S. littoralis* larvae, in comparison to the *B. bassiana* treatment (Lana *et al*., 2023), while *Trichoderma asperellum* showed high virulence against *S. frugiperda* larvae (Afandhi *et al*., 2022). Furthermore, combinations of *Trichoderma* and *B. bassiana* strains can act synergistically (Batool *et al*., 2020).

The mechanism of action of *Trichoderma* involves both direct and indirect attacks. In the direct mode, after the steps of adhesion, fixation, and germination, *Trichoderma* is able to release chitinolytic enzymes that attack the structure and permeability of the peritrophic matrix in the midgut of lepidopteran insects (Berini *et al*., 2016). Finally, insects can die due to nutrient deficiency from tissue damage or the effects of toxins, which are secondary insecticidal metabolites produced by *Trichoderma* (Dwisandi *et al*., 2024). It is worth noting that the effectiveness of *Trichoderma* against lepidopterans depends heavily on the host's developmental stage, with the larval stage being the most susceptible to infection and antifeedant action when lepidopteran insects are in the feeding phase, while intervention in pupae or adults is less effective (Kryukov *et al*., 2018; Shoukry *et al*., 2019; Yusri, 2023; Afandhi *et al*., 2022; Batool *et al*., 2020; Ghosh; Pal, 2016; Lana *et al*., 2023; Keshan *et al*., 2015; Koul, 2008).

The innate immune system of insects relies on both humoral and cellular responses (Lavine et al., 2002). Hemocytes are the main mediators of cell-mediated immunity in insects, including processes such as phagocytosis, nodulation, encapsulation, and melanization. In addition to cellular immunity, humoral immunity also plays a significant role in insect defense (Yi *et al*., 2014) and involves three main steps: a) recognition of pathogen-associated molecular patterns (PAMPs) in pathogens by the host's pattern recognition receptors (PRRs) (Medzhitov; Janeway *et al*., 2000); b) activation of regulatory pathways; and c) production of immune effectors, including cellular phagocytosis and molecular effectors such as antimicrobial peptides (AMPs) (Yi *et al*., 2014).

Some functions of these AMPs are well-characterized in insects. For example, Cecropins, isolated from *Hyalophora cecropia*, were the first microbial peptides isolated and fully characterized and have since been identified in lepidopteran, dipteran, and coleopteran insects. Cecropins cause lysis of Gram-negative and Gram-positive bacteria (Steiner *et al*., 1981; Yi *et al*., 2014) and, in some cases, can act against filamentous fungi (DeLucca *et al*., 1997; Cavallarin *et al*., 1998; Bulet *et al*., 1999; Shamakhi *et al*., 2019; Shahriari *et al*., 2021).

Attacin, isolated from the same moth species, has an antibacterial effect mainly against Gram-negative bacteria and filamentous fungi (Carlsson; Engstrom; Palva, 1991; Yi *et al*., 2014; Shamakhi *et al*., 2019; Shahriari *et al*., 2021). These peptides are generally rapidly induced after infection, characterizing them as immediate response genes in the insect immune system (Hultmark *et al*., 1983; Tanji; Ip, 2005). Several PRRs, including peptidoglycan recognition proteins (PGRPs), have been reported (Watson *et al*., 2005). PGRP was first purified from the hemolymph of the silkworm (*Bombyx mori*) (Yoshida *et al*., 1996; Michel *et al*., 2001), being fundamental for the activation of the Toll and Imd pathways in *Drosophila melanogaster* (Lemaitre; Hoffmann *et al*., 2007; Royet; Dziarski, 2007).

Insect innate immunity has been studied in *Drosophila melanogaster*, *Anopheles gambia*, and *Maduca sexta* (Hoffmann *et al*., 1999; Christophides *et al*., 2002). However, the mechanisms by which the *N. pronuba* immune system responds to these fungi have not yet been described.

The results obtained in this study, demonstrate that six hours after treatment (Figure 2), the expression of *Cecropin-1* and *Attacin* was more induced by Bb than by Ta. The expression of *Attacin* ($p = 0.026$) and PGPR-A ($p = 0.04$) was significantly higher in the Bb group than in the Ta group, suggesting a more immediate and coordinated response of these genes to Bb infection. This initial pattern is consistent with studies that showed a significant increase in the expression of *Attacin* and *PGPR-A* in other species, such as *Chilo suppressalis* (Lepidoptera: Crambidae), after infection by *B. bassiana* and *M. anisoplaie* and in *Helicoverpa armigera* (Lepidoptera: Noctuidae), after infection with *Candida albicans* (Shamakhi *et al*., 2019; Wang *et al*., 2010). The increase in AMPs may be the result of their continuous production by lepidopteran larvae (Shamakhi *et al*., 2019).

However, from 12 hours post-treatment (Figure 3), we observed a significant negative regulation (down-regulation) in the expression of all analyzed genes (*Cecropin-1*, *Attacin*, and *PGRP-A*) in both the Bb and Ta groups, compared to the control ($p < 0.0001$). This suppression of the immune response suggests that, responded primarily to the initial fungal infection in *N. pronuba*. Wang *et al*. (2010) also noted that, although *Cecropin-1*, *Attacin*, and *PGRP-A* are positively regulated in *H. armigera* after fungal infection, the magnitude and intensity of the response can vary over 3, 6, 12, and 24 hours after treatment with *C. albicans*. Additionally, the results of this work revealed a difference in the ability to suppress between the fungi, where *T. atroviride* suppressed the expression of the PGRP-A gene 4,391 times more than *B. bassiana* ($p = 0.027$). This finding is particularly relevant, as the entomopathogenic potential of

Trichoderma has been addressed in recent studies, where Afandhi *et al*. (2022) made the first report of the direct lethality of the fungus *T. asperellum* in *S. frugiperda* larvae. *T. hamatum* caused significantly higher larval mortality (85%) than *B. bassiana* (77%) in *S. littoralis* (Lana *et al*., 2023). In our study, *T. atroviride* caused a stronger suppression of the key pathogen recognition gene *PGRP-A* compared to *B. bassiana*, suggesting that these fungi differ in their capacity to modulate the humoral immune response of *N. pronuba* larvae.

The suppression of the immune response continued during the 24-hour evaluation after infection (Figure 4), but with a different behavior among the genes. *Cecropin-1* showed a small increase in expression with the Bb treatment, but without statistical significance. The negative regulation of *Attacin* persisted significantly for both treatments compared to the control group ($p = 0.0038$). For the *PGRP-A* gene, suppression remained significant only for the treatment with *T. atroviride* compared to the control ($p < 0.0001$) and was statistically different from the response to *B. bassiana* ($p = 0.0093$). The prolonged suppression of *Attacin* and *PGRP-A* indicates an extended modulation of the humoral immune response, though the actual impact on fungal development or host resistance cannot be determined from these data alone.

Analyzing the cellular immunity response is essential to correlate the molecular immune responses with the effective resistance of the insect and the virulence of the pathogen. However, in the present study, it was not possible to conduct cellular immunity analyses (e.g., hemocyte counts and survival tests) due to limitations in the colony maintenance, which after three successive attempts did not provide a sufficient number of larvae for complementary assays. We recognize that the absence of these analyses limits the direct extrapolation of the results to practical contexts. Therefore, to advance knowledge and improve applicability, future research should include integrated analyses that contemplate cellular and humoral immunity, as well as fungal virulence tests and evaluations under field conditions. Shamakhi *et al.* (2019) observed in *Chilo suppressalis*, after infection by *B. bassiana* and *M. anisopliae*, an initial increase in hemocytes (3–6 hours after treatment) occurring in accordance with the increase in gene expression of Cecropin and Attacin. After this period (12–24 hours after treatment), there is a gradual reduction. This result is consistent with the findings of suppression of the humoral immune response genes analyzed in this work, where immune system suppression occurs after the initial response, with the possibility of affecting both cellular and humoral immunity. The inclusion of cellular immunity analyses would strengthen the understanding of the global defense in *N. pronuba* against the fungal treatment of *Beauveria* and *Trichoderma*.

The expression dynamics of the genes in *N. pronuba* suggest an initial phase of

recognition and activation of the immune response (6 hours), followed by an active suppression of immunity by the pathogen (12 and 24 hours). The methodological difference between immersion and injection (Shamakhi *et al*., 2019; Wang *et al*., 2010) of conidia may explain the later response observed in our study compared to the analyzed works that start the response 3 hours after fungal inoculation. Since cuticular penetration is the first obstacle to be overcome by fungi in a field application context, we opted for this form of treatment to mimic a possible topical treatment performed in the plantation.

While the entomopathogenic activity of *Beauveria* is widely recognized and studied, that of *Trichoderma* is little explored. This work represents an advance in the knowledge of *N. pronuba*'s antifungal immunity, filling an important gap in the literature. These results, showing a significant suppression of the *N. pronuba* immune response by *T. atroviride*. They suggest that *B. bassiana* and *T. atroviride* have a considerable impact on the immune system of *N. pronuba* and deserve more investigation as a potential biological control agent.

5 CONCLUSION

Regarding the gene expression of humoral immune system-related genes *Cecropin-1*, *Attacin*, and *PGRP-A*, we observed that the intensity of gene expression to different pathogens varied throughout the hours assessed:

- Six hours after treatment, *Attacin* and *PGRP-A* were positively regulated following fungal infection, suggesting that these genes responded primarily to the initial fungal infection in *N. pronuba*. The gene response was more expressive with *Beauveria* treatment than with *Trichoderma.*
- Twelve hours after treatment, the response of all three analyzed genes was suppressed. The PGRP-A gene was the least expressed in response to the *Trichoderma* treatment, suggesting a transient modulation of the immune response in *N. pronuba* larvae.
- Twenty-four hours after treatment, *Beauveria* and *Trichoderma* maintained the suppression of the *Attacin* and *PGRP-A* genes, indicating prolonged modulation of the humoral immune response in *N. pronuba* larvae.
- The same gene can respond to different pathogens through different expression patterns.
- *B. bassiana and T. atroviride* have the potential to suppress the humoral immune response of *N. pronuba* following fungal treatment.

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ZHU, N.; ZHOU, J. J.; ZHANG, S. W.; XU, B. L. Mecanismos de fermentação de *Trichoderma longibrachiatum* T6 contra *Valsa mali* por meio da inibição de seu crescimento e reprodução, patogenicidade e expressão gênica. *Journal of Fungi*, v. 8, p. 113, 2022.

FINAL CONSIDERATIONS

O presente trabalho contribuiu de forma significativa para o entendimento das interações entre fungos entomopatogênicos e artrópodes de relevância veterinária e agrícola, explorando dois modelos biológicos distintos: o carrapato *Rhipicephalus microplus* e a mariposa *Noctua pronuba*. A partir de abordagens moleculares, microbiológicas e imunológicas, foi possível demonstrar que tanto a resposta imune do hospedeiro quanto a modulação de seu bacterioma intestinal podem ser influenciados pela dinâmica de infecção por fungos, refletindo a complexidade e a plasticidade das relações entre microrganismos e seus hospedeiros. No primeiro capítulo, a análise do bacterioma intestinal de *R. microplus* revelou que a infecção simultânea por *Metarhizium anisopliae* e *Anaplasma marginale* provoca alterações significativas na estrutura e funcionalidade da comunidade microbiana, com aumento da conectividade e da robustez das redes bacterianas. Esses resultados indicam que a microbiota intestinal desempenha um papel ativo na resposta do carrapato a múltiplos desafios infecciosos, podendo contribuir para a manutenção da homeostase e para a competência vetorial. Assim, a utilização de fungos entomopatogênicos como agentes de biocontrole em carrapatos deve considerar não apenas sua ação patogênica direta, mas também seus efeitos indiretos sobre o bacterioma e o equilíbrio fisiológico do hospedeiro. No segundo capítulo, o estudo da resposta imune de *N. pronuba* frente à exposição aos fungos *Beauveria bassiana* e *Trichoderma atroviride* evidenciou a capacidade desses organismos em modular diferencialmente a expressão de genes relacionados à imunidade inata. Enquanto *B. bassiana* promoveu uma ativação precoce de genes antimicrobianos, *T. atroviride* demonstrou um efeito supressor em momentos posteriores da infecção, sugerindo uma possível adaptação desse fungo não entomopatogênico às vias de defesa do hospedeiro. Esses achados ampliam a compreensão sobre os mecanismos de reconhecimento e defesa de insetos frente a diferentes tipos de fungos, apontando caminhos promissores para o desenvolvimento de estratégias mais específicas e eficientes de biocontrole. De forma integrada, os resultados obtidos reforçam que a aplicação de fungos entomopatogênicos vai além da simples indução de mortalidade em artrópodes-alvo, configurando-se como uma ferramenta multifuncional, capaz de interferir em redes ecológicas complexas que envolvem imunidade, bacteriota e patógenos associados. A incorporação desse conhecimento na formulação de novos produtos e programas de manejo integrado de pragas e vetores representa um avanço importante rumo a uma agricultura e pecuária mais sustentáveis, com menor dependência de compostos químicos e maior respeito à biodiversidade.

APPENDIX

Appendix 1

UFRRJ
Universidade Federal Rural do Rio de Janeiro

Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "ALIMENTAÇÃO ARTIFICIAL DE FÊMEAS DE Rhipicephalus microplus.", protocolada sob o CEUA nº 4832020623 (ID 002595), sob a responsabilidade de **Patrícia Silva Gôlo** *e equipe; Laura Nóbrega Meirelles; Thaís Almeida Corrêa; Victória Silvestre Bório* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro (CEUA/UFRRJ) na reunião de 02/10/2023.

We certify that the proposal "ARTIFICIAL FEEDING OF Rhipicephalus microplus FEMALES", utilizing 8 Bovines (8 males), protocol number CEUA 4832020623 (ID 002595), under the responsibility of **Patrícia Silva Gôlo** *and team; Laura Nóbrega Meirelles; Thaís Almeida Corrêa; Victória Silvestre Bório* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Veterinary Institute of Rural Federal University of Rio de Janeiro (CEUA/UFRRJ) in the meeting of 10/02/2023.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 10/2023 a 02/2026 Área: Parasitologia Animal

Origem: Laboratório de Quimioterapia Experimental em Parasitologia Veterinária da UFRRJ
Espécie: Bovinos sexo: Machos idade: 48 a 60 meses Quantidade: 8
Linhagem: Mestiço holandês Peso: 400 a 500 kg

Seropédica, 07 de maio de 2025

Prof. Dr. Fabio Barbour Scott
Coordenador da Comissão de Ética no Uso de Animais
Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro

Prof. Dr. Daniel de Almeida Balthazar
Vice-Coordenador da Comissão de Ética no Uso de Animais
Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro

BR 465, Km 7 / Campus da UFRRJ - Seropédica - Rio de Janeiro - CEP: 23890-000 - tel: 55 (21) 2682-3051
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CEUA N 4832020623

Appendix 2

UFRRJ
Universidade Federal Rural do Rio de Janeiro

Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Manutenção de colônia de Rhipicephalus microplus para avaliação do efeito de fungos entomopatogênicos no seu controle. ", protocolada sob o CEUA nº 6274250624 (ID 002857), sob a responsabilidade de **Patrícia Silva Gôlo** *e equipe; Thaís Almeida Corrêa; Adriani da Silva Carneiro Lopes; Laura Nóbrega Meirelles; Vânia R. E. Pinheiro Bittencourt* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro (CEUA/UFRRJ) na reunião de 02/09/2024.

We certify that the proposal "Maintenance of Rhipicephalus microplus colony aiming to evaluate the entomopatogenic effect of fungi for tick control. ", utilizing 30 Bovines (30 males), protocol number CEUA 6274250624 (ID 002857), under the responsibility of **Patrícia Silva Gôlo** *and team; Thaís Almeida Corrêa; Adriani da Silva Carneiro Lopes; Laura Nóbrega Meirelles; Vânia R. E. Pinheiro Bittencourt* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Veterinary Institute of Rural Federal University of Rio de Janeiro (CEUA/UFRRJ) in the meeting of 09/02/2024.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 10/2024 a 10/2029 Área: Parasitologia Animal

Origem: Laboratório de Quimioterapia Experimental em Parasitologia Veterinária da UFRRJ
Espécie: Bovinos sexo: Machos idade: 48 a 60 meses Quantidade: 30
Linhagem: Mestiço holandês Peso: 400 a 500 kg

Seropédica, 07 de maio de 2025

Prof. Dr. Fabio Barbour Scott
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