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Quantification and Comparative Analysis of Arachnid Microbiomes via 16S rRNA Sequencing

(*Pholcus phalangioides, Argyrodes argyrodes, Nephila senegalensis*)

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Dedication

To my father, Alan Jason Clifton, who carried me on his shoulders as a little girl, and who still carries me through every milestone with love, courage, and strength.

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Abstract

This study presents a first analysis of the microbiome of the haplogyne spider *Pholcus phalangioides* alongside the characterisation of two ecologically and phylogenetically distinct species of entelegyne spider: *Argyrodes argyrodes* and *Nephila senegalensis*. Using 16S rRNA metabarcoding, the study examines the diversity and composition of microbial communities within these spiders, including the identification of endosymbionts, with a primary focus on describing community structure and identifying taxa shared both intraspecifically and interspecifically. Quantitative metabarcoding data from *Pholcus phalangioides* exemplify the feasibility of sequencing microbes from within this type of as yet unstudied species, which will provide a valuable comparison to the more fully characterised microbiomes of *Argyrodes* and *Nephila.*

This research aims to establish a baseline for understanding how microbial associations may vary with spider host ecology. While primarily exploratory, the findings offer foundational insights into the potential ecological and evolutionary roles of host–microbiome interactions in arachnids as a whole. In summary, this study found the microbial communities to vary significantly between individuals and species; *Nephila* are highly dominated by the bacteria *Serratia,* which therefore makes their microbiomes less diverse, and *Argyrodes* individuals, at an intraspecific level, have differing compositions of the endosymbiont *Cardinium*. Additionally, this study, for the first time, quantified the sequencing reads of *Pholcus*, which helps to identify successful methodologies for uncharacterised and previously unsequenced organisms.

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This thesis is dedicated to my dad. Throughout my MRes, he has been bravely and quietly battling multiple myeloma. Despite enduring two autologous stem cell transplants and countless treatments, he has never stopped encouraging me and my sister to keep going, to believe in ourselves, and to never give up. One of the many things he's taught me is that *"nothing worth having comes to you easily,"* and those words have stayed with me through every setback and success. Without him, I would have simply given up. You are with me always, every step of the way. You are my grit and determination. You are my hero. This thesis is entirely for you, Alan Jason Clifton.

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List of abbreviations

- Polymerase chain reaction (PCR)
- rRNA Ribosomal RNA)
- P. *phalangioides* (*Pholcus phalangioides*)
- Cytoplasmic incompatibility (CI)
- *Drosophila* C Virus (DCV)
- COI gene (Cytochrome c Oxidase Subunit I)
- Quality control (QC)
- Principal Coordinates Analysis (PCoA)
- Operational taxonomic units (OTUS)
- Amplicon sequencing variants (ASVs)
- Thermostable DNA polymerase (Taq Polymerase)
- NanoDrop spectrophotometer (NanoDrop)
- Ribosome-inactivating proteins (RIPs)
- Deoxyribonucleic acid (DNA)

Chapter 1: Introduction

Arachnid and invertebrate microbiomes have demonstrated valuable applications in human health, including limiting zoonotic transmission, supporting cancer therapies, and serving as vectors for treating diseases such as malaria (Chakravorty et al., 2007; McKenna et al., 2008; Huttenhower et al., 2012; Luna-Ramirez et al., 2017; Sarkar et al., 2020; Moskwa et al., 2023; Cabezas-Cruz, 2023; Shi, Yu, and Cheng, 2023). While research on invertebrate microbiota is expanding, the landscape remains mostly unexplored and insufficient for arachnids. For *Pholcus* in particular, the species remains absent from microbiome research. *Pholcus phalangioides,* a synanthropic species with global distribution, provides a valuable model for investigating microbiome–host interactions across diverse environments. This study will explore intraspecific and interspecific comparisons between this and the other ecologically and phylogenetically distinct species: *Nephila senegalensis* and *Argyrodes argyrodes*. These species differ in web structure, prey selection, and ecological niche, allowing investigation of the relationship between microbiome composition and factors such as evolutionary or ecological divergence. This study will quantify the microbiome of *Pholcus phalangioides as* a first step in understanding the species' microbial community. By comparing the taxa identified here to microbes previously documented in other biological hosts, this work will establish a baseline for future novel ecological or functional analyses of arachnids and invertebrates alike.

The feasibility of using metabarcoding to sequence the microbiome of *Pholcus phalangioides* for the first time will be investigated, with a quantitative assessment based on the number of sequencing reads and the quantity of deoxyribonucleic acid (DNA). These data will be compared with microbiome characterisations of *Argyrodes argyrodes* and *Nephila senegalensis*, contributing to knowledge on the sequencing of previously uncharacterised organisms. Interspecific and intraspecific microbiome composition comparisons will be made from the full microbiome characterisations of *Argyrodes* and *Nephila,* with additional insights provided through bioinformatic diversity metrics. To contextualise this study, the following section outlines key background concepts in microbiome–host interactions and behavioural ecology, with a focus on how these may apply to arachnid systems.

Background and context

In the natural world, animals exhibit behaviours that often become fixed within populations because they enhance fitness, fecundity, growth, or survival, features that are sometimes related to the presence of microorganisms such as vertically acquired endosymbionts (Goodacre and Martin, 2012). In spiders, these behaviours are shaped by both intrinsic and extrinsic factors. Environmental variables such as pH, temperature, pollution, desiccation, and resource availability have been shown to influence behaviour and habitat selection (Bristowe, 1958; Marczyk et al., 1993; Canals et al., 2015) As a result, an emerging area of interest is the role of the microbiome, the community of bacteria, fungi, and viruses residing on or within a host, exploring if there are any interactions between the host and microbiome

that are capable of modulating invertebrate behaviour and life-history traits. In invertebrates, microbiota commonly inhabit the gut (Engel and Moran, 2013), skin (Dada et al., 2021), reproductive tissues (Werren et al., 2008), and internal organs (Hansen and Moran, 2011), where they can influence physiology, development, immunity (Dong, Manfredini and Dimopoulos, 2009), and behaviour (Teseo et al., 2019).

This thesis investigates whether such microbiome–host interactions extend to arachnids, with a particular focus on three spider species: *Pholcus phalangioides, Nephila senegalensis,* and *Argyrodes argyrodes*. While microbiome research in insect models such as *Lepidoptera, Diptera, Hymenoptera, and Coleoptera* is well established, comparable, varied and extended studies in spiders remain virtually absent. These three species offer contrasting ecological contexts, ranging from synanthropic to orbweaving lifestyles, providing a unique opportunity to characterise arachnid microbiomes and explore their potential ecological relevance. Building on these concepts, the next section reviews existing literature on invertebrate microbiomes and their evolutionary, ecological, and behavioural significance.

Literature review

Microbiomes in Invertebrates: Diversity, Composition, and Function

The microbiome comprises the community of bacteria, fungi, and viruses that inhabit an organism's internal and external environments. Some of these microorganisms are located intracellularly, while others occur extracellularly within tissues. In invertebrates, bacteria commonly colonise the gut, reproductive organs, and cuticle, where they can influence host physiology, metabolism, and reproduction. Microbiomes play a central role in regulating immunity, protecting against pathogens, and supporting essential functions such as digestion, nutrition, and development. Beyond immediate physiological effects, microbiomes also provide an evolutionary context for understanding host behaviour, ecological adaptation, and niche specialisation. Studies of microbiome diversity have transformed our understanding of host biology and evolution, revealing how microbial communities can shape phenotypes and mediate adaptation to environmental pressures. However, most research has focused on taxa of economic or ecological importance, such as pollinators including *Apidae* and *Vespidae*, while the microbiomes of arachnids remain comparatively underexplored.

Across arthropod taxa, microbiome composition is diverse and shaped by factors such as host phylogeny, ecology, diet, and life history strategy, as exemplified in subsequent sections below. Despite substantial variability across arthropod hosts, several dominant bacterial phyla occur consistently across groups, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Yun et al., 2014; Colman, Toolson and Takacs-Vesbach, 2012). For instance, arthropods such as bees (*Apis* spp.), flies (*Drosophila* spp.), and termites (*Isoptera*; *Reticulitermes* spp) form core microbiomes that form symbioses between microbiota and host, which can have different modes of transmission and acquisition via different mechanistic approaches, for example, diet, inoculation through the presence or absence of social contact (Engel & Moran, 2013; Kwong and Moran, 2016). On the other hand, some

arthropods, such as crustaceans and mites, demonstrate a more diverse microbiome structure, which is affected by extrinsic variables such as diet and habitat, which can imply that environmental influences can shape and manipulate the microbiome acquisition (Wang et al., 2019; Goffredi et al., 2023).

Furthermore, comparable studies highlight that there are similarities and comparable differences between arachnids and arthropods for example, the spider microbiome tends to be less diverse and complex than that of an insect, demonstrating predominance of certain taxa (*Wolbachia*, *Rickettsia*, *Cardinium*, and *Serratia)* (Goodacre et al., 2006; Vanthournout and Hendrickx, 2015; Sheffer et al., 2019), these taxa are most commonly denoted as maternally inherited endosymbionts, as opposed to free-living organisms, suggesting that symbiosis and reproductive manipulation is likely to be of higher importance in spiders than it is for insects (Kumar et al., 2020; Tyagi et al., 2021).

Additionally, further studies conducted on different kinds of spiders (orb-weavers, jumping spiders) also suggest that microbiome composition varies between spider species, geographic location, and tissue type, suggesting that variables affecting spider microbiomes are likely to be modulated extrinsically and intrinsically (Tyagi et al., 2021), through mechanisms that are distinctly different for each species e.g. (habitat, geographic location, lifestyle, diet). However, at present, it is difficult to quantify the functional contributions of the arachnid microbiome to phylogeny, ecology, behaviour, and immunity, as they remain largely uncharacterised (Zhang et al., 2018). Together, these findings reveal that while invertebrate microbiomes are diverse and ecologically significant, arachnid-associated microbial communities remain an underexplored frontier. In particular, the dominance of vertically transmitted symbionts such as *Wolbachia* and *Rickettsia* hints at unique evolutionary dynamics within spiders. These patterns set the stage for further exploration of endosymbiotic relationships and their co-evolutionary implications across arthropods.

Endosymbiotic Relationships and Co-evolution in Arthropods

The synergistic interaction between *Wolbachia* and *Spiroplasma* has been shown to enhance survival in fly larvae exposed to parasitic nematodes (*Steinernema carpocapsae*) but not against xenic nematodes grown in uncharacterised microbial environments (Ahmed, 2015; Yadav et al., 2018). Notably, *Spiroplasma*, another heritable endosymbiont, has been identified in at least sixteen *Drosophila* species (Haselkorn, 2010). While its exact phenotypic effects are not fully characterised, *Spiroplasma* produces ribosome-inactivating proteins (RIPs) that protect hosts from viral and parasitic threats. However, in the absence of such viral threats, continued RIP expression can reduce fly lifespan and increase male embryo mortality (Garcia-Arraez et al., 2019).

Extending beyond insect models, the first full microbiome characterisation of the wasp spider *Argiope bruennichi*, which excluded endosymbionts previously characterised in invertebrates and arthropods, was a study led by Sheffer et al. (2019). The study measured diversity amongst different tissue types from the wasp spider, which included prosoma, hemolymph,

book lungs, ovaries, silk glands, midgut, and faecal pellets. Sheffer et al. (2019) also revealed significant intraspecific and interspecific variation of the wasp spider microbiome, and the same amount of variation was also observed amongst different tissue types. Notably, the study identified a previously uncharacterised bacterial symbiont that is present amongst all tissue types of geographically distinct populations. The novel symbiont was affiliated with the *Tenericutes* phylum despite low sequence identity. Due to the absence of genetic matches with known symbionts, the symbiont was considered a novel clade. The detection of this symbiont in wasp spider offspring strongly suggests vertical transmission, marking an important discovery in arachnid symbiosis and microbial inheritance (Sheffer et al., 2019).

The identification of a novel, vertically transmitted symbiont in *Argiope bruennichi* raises important questions about the ecological and physiological factors that shape endosymbiont persistence across generations. Among these, temperature emerges as a critical environmental variable influencing symbiont abundance, activity, and transmission dynamics.

Endosymbiont Temperature and Transmission

Temperature is a key environmental factor influencing the abundance and activity of microbial symbionts. While it is well established that elevated temperatures can damage or kill free-living bacteria, the dynamics within endosymbiotic systems are more complex and context-dependent. In invertebrates, the thermal environment, including both microhabitat selection and exposure to fluctuating temperatures, can significantly affect the stability and function of host-endosymbiont relationships.

These effects extend to host behaviour, symbiont replication, and long-term infection persistence. Numerous studies (Corbin et al. 2017; Martins, Cássia Siqueira César and Cogni, 2023) have reported that endosymbiont titre is sensitive to temperature changes, though the direction of this effect varies. In some systems, higher temperatures correlate with increased symbiont abundance (Stillson et al. 2025**)**; in others, particularly involving intracellular bacteria like *Wolbachia*, elevated temperatures reduce titre (Zhang and Moran, 2019; Barman et al. 2023). These outcomes depend on the host species, developmental stage, symbiont strain, and environmental context.

Wolbachia is one of the most widespread and well-studied endosymbionts in arthropods and invertebrates alike (Kent and Bordenstein, 2010; Zug and Hammerstein, 2014; Lorenzo-Carballa et al., 2019; Lucek et al., 2021). It is known for its capacity to influence host reproduction (Bi and Wang, 2019), transmission efficiency (Łukasz Kajtoch et al., 2019), and microbial community structure (Morgane Ourry et al., 2021). Although much of the foundational work has focused on insect models such as *Drosophila*, these findings offer valuable insights into the mechanisms that may also shape microbial dynamics in other arthropod groups, including spiders. This section reviews the key mechanisms by which *Wolbachia* interacts with its hosts, particularly how environmental variables such as temperature affect its transmission, titre, and associated phenotypes. These dynamics are essential to consider when interpreting microbiome data from spider species, where similar symbionts may play parallel ecological or physiological roles.

Wolbachia is a maternally inherited endosymbiont that has been shown to influence host thermoregulation, development, and reproductive success (Truitt et al., 2018; Hague, Caldwell and Cooper, 2020; Osorio et al., 2023). One key mechanism by which *Wolbachia* exerts influence is by altering host behaviour. Hauge et al. (2020) hypothesised that *Wolbachia*-induced behavioural changes mediate *Drosophila* temperature preferences. This was supported by findings showing that *Wolbachia*-A infected flies preferred cooler environments compared to uninfected controls. Conversely, divergent B-group strains such as wMau were associated with warmer temperature preferences. Temperature also has a direct effect on *Wolbachia* titre within the host. Studies (Zhou et al., 2019; Hu et al., 2023) have shown that elevated temperatures, particularly between 25°C and 31°C, lead to reduced *Wolbachia* titres, negatively impacting *Drosophila* development, survival rates, and reproductive success. This includes lower egg hatching rates, reduced vertical transmission, and smaller body size. Despite this, some B-group strains such as wSh and wTei exhibit increased titres shortly after exposure to lower temperatures, suggesting a temperature-sensitive replication mechanism.

Imperfect vertical transmission, where *Wolbachia* is not reliably passed from mother to offspring, has also been linked to environmental factors. Marjolein Bruijning et al. (2020) and Hauge et al. (2024) noted that latitude, host genetic background, and low ambient temperatures are key contributors to reduced maternal transmission efficacy. This variability in transmission correlates with changes in *Wolbachia* titre, particularly under stress conditions such as temperature extremes. Cytoplasmic incompatibility (CI) is another significant *Wolbachia*-induced phenotype. It occurs when infected males mate with uninfected females, resulting in embryo mortality due to impeded paternal chromosomal condensation (Hochstrasser, 2022). The expression of CI is modulated by both intrinsic factors, such as host age and sex, and extrinsic factors like temperature (Ritchie et al., 2022).

Studies by Seyede Fatemeh Nasehi et al. (2021) and Ritchie et al. (2022) demonstrated that while wildtype CI efficiency decreases with reduced *Wolbachia* titre at higher temperatures, transgenic flies maintain CI even under thermal stress. In parasitoid wasps like *Habrobracon hebetor*, Cif gene expression (especially CifA), which is known to cause CI, was found to be higher in males, linking gene expression to CI induction. Other species, such as the flour beetle (*Tribolium confusum*), showed unaltered CI despite reductions in *Wolbachia* titre at elevated temperatures (Yeganeh Gharabigloozare and Bleidorn, 2022). However, fertility rates declined, likely due to the decreased symbiont load. Similarly, super parasitised female wasps exhibited reduced lifespan and fecundity, suggesting a trade-off between *Wolbachia* transmission and host fitness.

Zhou et al. (2019) and Guo et al. (2023) further reported that temperature increases led to reduced *Wolbachia* titres and skewed sex ratios. Guo et al. (2023) also demonstrated that antibiotic treatment reversed *Wolbachia*-induced thelytoky in *Trichogramma*, restoring arrhenotoky and increasing microbial diversity. Therefore, the combination of antibiotics and high temperatures suppressed CI and male-killing effects observed in the host. Environmental stressors beyond temperature can also modulate *Wolbachia's* impact and influence on host

physiology. Wiwatanaratanabutr and Kittayapong (2006) found that insecticide exposure did not affect *Wolbachia* titre but did reduce wing size and survival rates in *Aedes albopictus*. Their later study confirmed that *Wolbachia* titres in *Aedes albopictus* decreased at higher developmental temperatures across life stages and that larval crowding negatively impacted adult size and symbiont density (Wiwatanaratanabutr and Kittayapong, 2009).

Furthermore, Chrostek et al. (2021) highlighted the potential for leveraging *Wolbachia's* temperature sensitivity in public health. By using *Drosophila* as a model, they demonstrated that antiviral protection conferred by *Wolbachia* against *Drosophila* C Virus (DCV) is temperature-dependent. Flies reared at 18°C exhibited significantly reduced antiviral protection compared to those reared at 25°C, despite the general trend of *Wolbachia* titre declining with increasing temperatures. These findings have implications for vector control strategies targeting viruses such as the dengue virus and the Zika Virus (Talapko et al., 2019; Chilakam et al., 2023; ECDC., 2024; Roiz et al., 2024). In summary, temperature plays a central role in modulating *Wolbachia's* replication, transmission efficiency, modulation of host physiology and behaviour, as well as altering observed host phenotypes in combination with other extrinsic variables. While low temperatures may enhance titre in some strains of *Wolbachia,* elevated temperatures generally reduce *Wolbachia* load, impairing both vertical transmission and endosymbiont-induced phenotypes like cytoplasmic incompatibility (CI). Therefore, understanding these dynamics is essential for both ecological studies on invertebrates and spiders, as well as critically important for the potential application of *Wolbachia in* vector control programs

Endosymbiont Transmission Mechanisms

Given the importance of maternal transmission in endosymbiont inheritance, recent work has focused on how temperature affects *Wolbachia* localisation and proliferation within reproductive tissues. Hauge et al. (2024) reported that infected ovaries and eggs showed significant variation in the distribution of *Wolbachia*, which was transmitted from host offspring across different temperature parameters. The data concluded that between 20°C and 25°C, *Wolbachia* titre and maternal transmission differ in efficacy, leading to decreased abundance, no change in abundance, and increased abundance when exposed to colder temperatures.

Radousky's work in 2023 outlined how *Wolbachia* colonies interact with other cells to proliferate further through their host's bodies, particularly those in the reproductive system, and how this influences vertically closely related *Wolbachia* strains. Those strains tend to proliferate in the reproductive systems of *Drosophila* via very similar mechanisms, such as posterior localisation. Similarly, when discussing the transmission and frequency distribution of endosymbionts, it is also essential to consider that *Wolbachia* accumulates itself differently throughout the reproductive system; Radousky et al. (2023) identified that *Wolbachia* distribution is found in different relative abundances in oocytes. In addition, the same study discovered that *Wolbachia* within oocytes can access the germline from other nearby somatic follicle cells, which enables *Wolbachia* to maintain both lines of transmission: germline-to-germline transmission or germline-to-somatic transmission routes. This, in turn, allows

Wolbachia to successfully colonise a broader spectrum of host species and cause maximum spread.

A similar point of interest would be that the authors also found three critical differences in the distribution of *Wolbachia* throughout the female oocyte, including a tight clustering of *Wolbachia* titre at the posterior pole plasm, a concentrated titre at the posterior pole, and finally, a titre found throughout the oocyte. However, quantitative data suggested the titre of *Wolbachia* found was very diminished, if any was present at all. Moreover, the results also indicated that closely related strains of *Wolbachia* tend to use the same transmission methods to invade the reproductive systems and neighbouring cells of *Drosophila* (Radousky et al., 2023). While *Wolbachia* is primarily maternally (vertically) transmitted *(*Hertig, 1936), horizontal transmission may also occur, though this is less common and not fully understood (Pietri, DeBruhl and Sullivan, 2016). The presence of multiple *Wolbachia* strains in different species and life stages complicates assumptions about exclusive vertical inheritance.

Mechanisms Driving Variation in Arthropod Microbiomes

The availability of high-throughput sequencing has led to increased insights into the microbiomes of key pollinators, with honeybees and solitary bees providing contrasting models of microbial acquisition and diversity. Honeybees *(Apis spp.)* have a well-defined core microbiome composed of nine bacterial taxa, largely acquired through social contact within colonies. These microbes are implicated in immune defence, development, and resistance to pathogens. Disruption of the gut microbiome negatively impacts overall bee health and colony resilience (Kotch et al., 2013; Raymann and Moran, 2018).

In contrast, solitary bees lack this stable core microbiota due to their non-social lifestyle; the microbiome is more variable and heavily influenced by environmental exposures like pollen and nesting substrates (Voulgari-Kokota et al., 2019; Powell et al., 2023). While the bacterial diversity is greater, the functional stability is less clear. One study reported that both vertical and horizontal transmission modes can occur in solitary and social bees, depending on the species and environmental context (Kapheim, Johnson and Jolley, 2021). In addition, Boff and colleagues argue that the landscape a solitary bee colonises is a cofounding variable to the influence that fungicides have on microbiome composition. The study found that solitary bees from anthropogenic landscapes often show reduced microbial diversity, while individuals from more natural environments may harbour richer microbiota (Boff et al., 2021), indicating that the environment can mitigate the effects caused by fungicides, which reduce microbiome heterozygosity. Similarly, it was reported by Porras et al. (2024) that fungicides used in pre-bloom and blooming flowers were detrimental to solitary mason bees by showing reduced microbiome diversity in all doses in the fungicide-treated group. On the contrary, solitary bees exposed to untreated pollen had a higher microbiome diversity. In addition, the study found that solitary bees exposed to fungicides were at an increased risk of mortality and suffered from low weight gain across larvae, as well as a sex ratio skew, favouring males (Porras et al., 2024).

A study conducted on two geographically distinct social wasps, the *Vespula pensylvanica*, demonstrates that this species of wasp has a simple and core microbiome composed of bacteria, including *Fructobacillus, Fructilactobacillus, Lactococcus, Leuconostoc,* and *Zymobacter*, which is mainly acquired via horizontal transmission methods such as food and environment. However, the wasp is often infected with the Moku Virus and dominated by various endosymbionts (Rotham et al., 2021). In addition, the study also examined the possibility that the wasp microbiome composition and the Moku virus could interact with each other in some way. However, Rotham et al. (2021) ruled out any interactions between the two. Despite this finding, the study identified that the wasps often harboured bacterial bee taxa, suggesting that *Vespula pensylvanica* could be a facultative vector for viral and bacterial infections, suggesting trophic transmission strategies.

Furthermore, Edward and Bordenstein's study (2019) on parasitic wasps (e.g., *Nasonia)* demonstrated that mismatched microbiota can modulate phenotypes and overall success, such as larval growth, pupation rate and overall adult fecundity of parasitic wasps. They showed this by comparing two groups: the interspecific microbiota and the intraspecific microbiota found in the wasps to identify any differences. Edward and Bodenstein (2019) concluded that gut microbiota transplants in parasitic wasps can impair larval growth and adult survival, although male fertility remained unaffected.

Wolbachia, a widespread endosymbiont in *Hymenoptera*, can reshape host microbiomes and even drive reproductive mode (Guo et al., 2023). In asexually reproducing wasps, *Wolbachia* infection is associated with reduced microbial diversity compared to their sexually reproducing counterparts (Brinker et al., 2022), supporting the idea that endosymbionts can streamline microbiomes toward homogenisation when the microbiome is less diverse due to *Wolbachia's* presence (Duan et al., 2020; Henry et al., 2024). In Wang's (2020) study, it was found that wasps adapt to environmental pressures such as herbicides. The study also proved that wasps exposed to atrazine developed resistance to the herbicides by altering their gut microbiota with microbes known for degrading Atrazine. Additionally, the study highlighted that modulation of the gut microbiome composition is a trait passed on to successive generations with increased penetrance denoted over time, which suggests not only microbiome-mediated adaptation but also the evidence of vertical gut microbiome transmission.

These findings in wasps underscore the adaptive potential of microbiome modulation under environmental stress and the heritability of microbial traits across generations. Extending this perspective, recent work on hoverflies reveals how developmental stage and trophic interactions further shape microbiome composition and transmission routes.

Developmental and Trophic Mechanisms of Microbiome Variation: Hoverflies

The hoverfly *Episyrphus balteatus* maintains a core microbiome of *Proteobacteria* and Firmicutes that stays mostly consistent among both sexes and life stages. However, some microbiota vary in abundance across life stages (Wang et al., 2024). For example, *Enterococcus silesia cus* and *Morganella morganii* dominate in the larvae and pupae developmental stages, while *Cosenzaea myxofaciens* primarily dominates during adulthood.

Notably, the study also found that several endosymbionts commonly found in hoverflies are also facultative symbionts in aphids, including *Serratia*. This suggests interspecific microbiome sharing and horizontal transmission from the aphid as prey to the hoverfly host. Additionally, symbiont presence in hoverflies demonstrates the expected sexual bias phenotype that commonly occurs within species infected by endosymbionts. These variables also influenced the species' success, evidenced by the newly emerged adults exhibiting a 25% mortality rate, which was hypothesised to correlate with disrupted microbiome composition (Chang et al., 2024). Cumulatively, these findings imply a potential relationship between microbiome stability, facultative symbionts, and key life history traits such as survival, fecundity, and longevity.

Behavioural and Ecological Modulators of Microbial Communities

While developmental stage and trophic interactions clearly shape microbiome composition in species like hoverflies, behavioural and ecological factors also play a critical role in modulating microbial communities. *Drosophila* is a widely used model organism, particularly in genetics research, because it is easy to maintain, has short generation times, and has a simple physiology (Tolwinski, 2017). These characteristics have made the flies central to studies, making discoveries in microbiome-host interactions. For example, Jia et al. (2021) explained that germ-free male flies showed significantly reduced aggression compared to their conventionally reared counterparts; from this, it can be inferred that the microbiome can modulate social behaviours. Despite this finding, the study declared no changes were observed in the flies' courtship or locomotion behaviours, and overall fecundity remained unaffected, raising questions about the specific behavioural pathways influenced by microbial presence.

Diet is also a key factor in modulating the microbiome and behaviour. Ferreira and Caetano (2023) explored how the composition of a fruit fly's microbiome is indicative of what they had previously consumed within their diet. The study denoted that both yeasts and bacteria found within the microbiome come together to provide nutrient-rich food that supports larval growth and development. Ferrira and Caetano (2023) demonstrated that *Drosophila* development and overall success are mediated by diet and subsequent microbiome composition. They also validated the fact that microbes such as bacteria and certain types of yeast that compose the microbiome can interact with each other and have mediating effects upon larval nutrition and development.

Beyond dietary influences on microbiome composition and larval development, strain-level variation in endosymbionts such as *Wolbachia* also contributes to microbial dynamics and host physiology in *Drosophila.* Detcharoen and Nilsai (2022) studied two different types of *Drosophila*: *Drosophila ananassae* and *Drosophila simulans*. They identified two novel strains of *Wolbachia*: (wMalA and wMal), distinct from the commonly found wRi strain in related species, arthropods in the Thai Peninsula. The presence of both of these novel strains was interesting in the absence of any other endosymbiont infections. These findings indicate that the more novel strains of *Wolbachia*, especially in tropical regions, could prevent co-

infection with other endosymbionts, although the mechanisms behind this are still not fully understood (Detcharoen and Nilsai, 2022).

Beyond insect systems, comparable symbiotic relationships between bacterial endosymbionts and the modulation of host behaviour might still be possible in arachnids. Work conducted by Goodacre and Clifton (2023, unpublished) suggested a possible association between the causal relationship of intraspecific behavioural variation observed in the *Pholcus*-specific whirling behaviour and the presence or absence of endosymbiont (*Rickettsia* and *Wolbachia)* infections in UK populations of *P. phalangioides*. Subsequently, their preliminary study is suggestive of the complex and largely understudied interactions between intra-specific host behavioural variation and microbial infections, which provides a foundation for further investigation on microbiomes and their potential influence on behavioural modulation.

While behavioural responses are often shaped by microbial associations, they do not occur in isolation. Behaviour is closely correlated with host physiology and life-history strategies, which also shape and are shaped by microbiome structure, which is the focus of the next section.

Physiological and Life History Factors Shaping Microbiome Structure

Behavioural Thermoregulation and Environmental Adaptation

Environmental factors also play a role in the modulation and transmission of fly microbes and symbionts. Bykov et al. (2019) found that populations of *Drosophila* that survive after overwintering in Palearctic regions are more likely to transmit *Wolbachia* vertically than horizontally, although a secondary finding was that infection rates did not correlate with any latitudinal or longitudinal patterns. Furthermore, Simhadri et al. (2017) reported that *Wolbachia* negatively affects the diversity of the host gut microbiome titres in laboratory-reared *Drosophila*, despite the endosymbiont not residing in the gut lumen. This suggests that there is an indirect relationship or influence between gut microbiome community modulation and *Wolbachia* infections present within other areas of the host's body.

Another important study of how microbiomes mediate fruit flies is Hague et al.'s (2021) work, which assessed how *Wolbachia* influence locomotion across nine *Drosophila* species infected with fourteen different *Wolbachia* strains. They reported that *Wolbachia* altered locomotor activity in six host genotypes, with the direction of these effects varying unpredictably and often in a sex-dependent manner. The authors hypothesised that variation in symbiont titre and tissue localisation, particularly within the central nervous system, could modulate these observed behavioural changes.

Cockroaches, particularly *Shelfordella lateralis* (Renelies-Hamilton et al., 2021), represent an interesting case when it comes to arachnid microbiome transmission. The study explored that although cockroaches harbour a stable microbiome that comes from vertical transmission, subsequent social inoculation modulates the microbiome more strongly and drives host-taxa effects on which microbes establish. One of the results of this work proved that there are similarities between maternal egg case microbiota and offspring microbial profiles,

suggesting some degree of vertical transmission. This seemingly protects young cockroaches with microbes termed ‘stabilisers ' that take priority over the microbiome and subsequently stabilise it from colonisation by opportunistic microbes (Renelies-Hamilton et al., 2021). The study highlighted that inoculation and associated horizontal transmission drive communities towards different stable states and the factors that affect this appear to be extinction events and colonisation (Renelies-Hamilton et al., 2021). However, a factor that also modulates different stable microbiomes within cockroaches is diet, and the study concludes that diet shapes the functional capabilities of these different microbiomes.

In contrast, Tinker and Ottesen (2016) studied *Periplaneta americana*, an omnivorous cockroach, and found a stable and diverse core microbiome located in the hind-gut. They observed low variance intraspecifically, and this diversity did not significantly shift in response to dietary changes (Tinker and Ottesen, 2016), in comparison to observations in mammals, where diet is a major modulator of the gut microbiome (David et al., 2013; Mansour et al., 2021; Gregor et al., 2021; de Jonge et al., 2022). Another point to note would be that Tinker and Ottesen (2016) also identified that, despite laboratory reared cockroaches and wild-caught cockroaches having the same hind-gut microbiome, a difference observed between wild-type and lab-reared cockroaches was that the wild cockroach had a higher diversity of low-abundance microbes. In comparison, lab-reared cockroaches harboured less diversity amongst high-abundance microbes. Altogether, this study may suggest that the cockroach hindgut may be inherently more resilient or modulated by multiple factors other than just diet.

Thermoregulation is critical for ectothermic organisms such as spiders. Rao and Mendoza-Cuenca (2016) demonstrated that colour polymorphisms in orb-weaving spiders confer physiological advantages in managing body temperature under ultraviolet light exposure. Specifically, white-morphed individuals exhibited more effective thermoregulation than yellow-morphed counterparts, cooling down more efficiently without the need to relocate. Avoiding relocation is ecologically significant because building a new web is energetically costly, making this a notable example of a life history trade-off. Orb-weavers have adapted elongated body morphologies as a response to increased environmental temperatures, potentially improving heat dissipation and prolonging survival under thermal stress (Ferreira-Sousa et al., 2021). In contrast, smaller-bodied individuals with less surface area may be more vulnerable to heat-related mortality.

In 1974, there was very little literature to prove or disprove that arachnids can modulate their behaviour in favour of thermoregulation induced by extreme climates for example, Humphreys (1974) determined that Wolf spiders prefer climes higher than the ambient air temperature, the study also observed that the spiders were more active on clear, sunny days and mediated their movement in conjunction with shade or sun, with females incubating their egg sacs on their spinnerets in direct sunlight. Interestingly, and somewhat contradictory to the results observed in the same study, Humphreys (1974) reported no evidence of the burrowing spider exhibiting active behavioural thermoregulation and instead acknowledged

that freedom of movement was likely the root cause of the observations made during the study. Since 1974, studies have examined the potential correlation between behavioural modulation and thermoregulation in burrowing spiders, demonstrating that spiders are capable of employing strategies to prevent them from overheating in extreme desert environments (Humphreys, 1975; Humphreys, 1978; Lubin and Henschel, 1990; Steves, Berliner and Pinshow, 2021) an example of this would be *Seothyra* spiders in the Namib Desert can hunt in temperatures above 65°C, they do this by moving between hot sand and the retreat of a cooler burrow (Lubin and Henschel, 1990). The same study highlighted that spider showed indications of thermal stress at temperatures of 49°C when restrained from entering the cooler burrow. This collectively demonstrates that spiders can increase their fitness by altering their behaviour to increase the effectiveness of physiological thermal regulation mechanisms in extreme temperatures.

Polymorphisms and Polyphenism in Life-History Strategies

Invertebrates often demonstrate polymorphic behaviour and phenotypes associated with traits such as dispersal and migration. Polymorphisms can occur without observable morphological variation or can coincide with morphological or physiological changes that align with life history strategies, such as delaying reproduction in favour of dispersal or vice versa. For example, some individuals may retain traits beneficial for dispersal, while others may express traits that promote fecundity once movement ends (Dingle, 1996; Dingle, 2014). Such plasticity ensures that populations can exploit varying ecological niches over time.

Physiological Adaptations and Their Potential Influence on Microbiomes

Whirling, in which a spider vibrates or gyrates its whole body rapidly in response to a disturbance Heuts et al. (2001), could be considered a risk-engaging behaviour because the movement consumes finite energetic resources that may not be readily replenished, especially in natural environments where finite resources are scarce. Here, it is presented that the behaviour known as whirling in *Pholcus* spiders may represent a polymorphic life history trait; the behaviour is ubiquitous amongst individuals and shows wide-scale heterozygosity intra-specifically, for example, long-duration and short-duration whirling Heuts et al. (2001).

Here, it is argued that whirling might be a risk-engaging behaviour due to the trade-off that the spider makes between survival and the energy consumed to produce whirling. This is because whirling is conducted by utilising energy from finite resources. Evidence from arachnids shows that spiders modulate their life-history strategies in response to resource availability. An example of this would be that Linyphiidae's life history traits are influenced by the availability and abundance of finite resources such as food (Bonte, 2015). Bonte (2015) found that spiders choose whether to use silk spinning for predator escape and dispersal or upregulate energy into other life history traits, such as overall fecundity and survival. The study examined this by testing two groups: one with unlimited access to food and the other under dietary restriction, with both groups having their dragline spinning induced under natural conditions. It was concluded that strong trade-offs between dragline spinning, fecundity, and survival were

present. Dragline spinning had a detrimental effect on egg sac production, and post-silk weaving was also reduced. These findings were most likely confounded by nutritional deprivation, but nonetheless, the trade-offs were still observed.

Pholcus belongs to the infraorder araneomorphae, a diverse group of spiders with forward-facing, pinching chelicerae adapted to diverse predatory strategies (Huber, 2011). Unlike mygalomorph spiders (e.g., tarantulas), which have vertically oriented chelicerae, araneomorphs also differ in respiratory strategies and lifespan (Schmitz, 2015; Singh & Singh, 2020). *Pholcus* possess a tracheal system and a single pair of book lungs, allowing greater metabolic flexibility but typically shorter lifespans. In this species, males have larger lung volumes than females, though respiration is dominated by pulmonary function (Foelix, 2011). Additionally, other intrinsic and extrinsic factors have been proven to modulate arachnid metabolic rate, such as sex, finite resource availability, phylogeny, temperature, hunting style, presence of tracheal respiration, and life cycle duration (Schmitz, 2016). Another point to note on this topic is that mygalomorphs also vary in body and leg size compared with araneomorphs; araneomorphs tend to have longer and more slender legs alongside a smaller body size (Raven,1985; Sharma, Singh and Singh, 2020; Singh and Singh, 2022), as observed in *Pholcidae.* Taken together, the literature suggests that both intrinsic physiological traits and extrinsic factors shape how host–microbiome relationships are established and modulated. Taken together, these findings highlight how physiological, behavioural, and ecological traits can mediate microbiome composition across invertebrates. To explore these relationships empirically, this study focuses on three phylogenetically and ecologically distinct spider species that differ in their ecology, morphology, and behaviour.

Study species

This study focuses on characterising the microbiome of two spider species where sequence data had already been generated (*Argyrodes argyrodes and Nephila senegalensis)* and taking the first steps to do the same in *Pholcus phalangioides*, reaching the stage of generating metabarcoding sequencing reads. Taken together, these spiders were selected to be included in this study for their contrasting ecologies, morphologies, and behaviours. These three distinct arachnid species provide a useful framework for investigating microbiome variation across arachnid taxa.

Synspermiata

Knowledge of arachnid morphology and taxonomy is expanding all the time, especially with advances in microscopy and evolutionary genetics. Therefore, it is no surprise that over time, the clade to which the species *Pholcus phalangioides* belongs has been subject to scrutiny. As a result of the observed simplified genital morphology, the species was analysed phylogenetically to determine if the species can be categorised under the more refined clade ‘Synspermiata’, as many of the previously known haplogynae are now being reclassified into the clade Synspermiata. This is important because it helps to distinguish differences between

phylogenetically distinct lineages (monophyletic or polyphyletic clades), a distinction that directly influences how ancestry may relate to host-microbiome relationships observed during this preliminary study.

In 2014, Michalik and colleagues hypothesised that Synspermiata is a clade within the araneomorph spiders that consists mainly of what was formerly known as the haplogyne spiders (Michalik et al, 2014). The female Synspermiata spiders have simplified genitalia, and the males have a categorised arrangement of sperm, known as synspermia, whereby multiple sperm cells are compiled into one common specialised membrane (Alberti and Weinmann, 1985; Michalik et al., 2004). In addition, females and males both have a simplified reproductive system when compared to their counterparts, araneomorph spiders.

Pholcus phalangioides

Pholcus phalangioides is a haplogyne spider that spans every continent except Antarctica. They can be found in subtropical regions but are also found in tropical regions (Huber, 2011). Some species of *Pholcus* have been proven to be synanthropic, meaning they rely on living near humans for successful colonisation of their chosen niche. The reproductive biology of *Pholcus* has been extensively studied, with particular attention paid to the male reproductive system (Michalik and Uhl, 2005) and the possibility of sperm mixing (Uhl, 1998) and courtship rituals between males and females (Hoefler et al., 2010).

Pholcus form part of a large group of spiders called the araneomorphs. This group of spiders share a similar morphology in that they have distinctive chelicerae because they are a particular shape and are kept in a specific orientation (Coddington, 2005). The chelicerae in *Pholcus* point forward diagonally and cross over in a pinching action (Huber, 2011). In contrast, tarantulas, which belong to the Mygalomorphae infraorder of spiders, have chelicerae typically shaped to point downwards (Lüddecke et al., 2021). The differences in chelicerae orientations between spider infraorders demonstrate how evolution has equipped arachnids with distinct mechanisms to hunt and handle different kinds of prey, potentially contributing to differences observed in the acquisition of microbiomes. Additionally, morphological differences within the infra-order of species being investigated could also offer an explanation for any contaminants observed in the spider specimens, particularly spider abdomens.

Pholcus phalangioides also exhibits a distinctive behaviour known as whirling, in which the spider rapidly oscillates its body in a circular or pendular motion when disturbed. This movement blurs the spider's outline and is thought to deter visual predators such as salticid (jumping) spiders (Heuts et al., 2001; Harland, Li and Jackson, 2012). The behaviour is energetically costly and appears to be triggered primarily when the spider is under threat. Both short and long duration whirling have been reported, with longer bouts associated with predator encounters. In this thesis, individuals that perform this behaviour following light fingertip stimulation are referred to as whirlers (+), and those showing no response are referred to as non-whirlers (–).

Nephila senegalensis

Nephila are large, orb-weaving spiders known for their strong, golden silk and open web architecture. They inhabit warm, outdoor environments and exhibit sexual size dimorphism, with females being significantly larger (Selden, Shih and Ren, 2011). *Nephila* 's outdoor, solitary lifestyle and complex web-building behaviour contrast sharply with *Pholcus's* indoor dwelling and irregular webs. These ecological and behavioural differences may correspond to distinct microbial communities, shaped by environmental exposure and prey diversity. *Nephila* spiders are particularly well-known for their orbweaving behaviour (Selden, Shih and Ren, 2011), and exhibit life history traits that are notably responsive to environmental conditions (Lowe, Wilder and Hochuli, 2017).

Argyrodes argyrodes

The Argyrodes species are kleptoparasitic spiders (Agnarsson, 2003) that live in the webs of larger hosts such as *Nephila*, stealing prey rather than hunting directly (Grostal and Walter, 1997; Whitehouse et al., 2002) This unique ecological niche: living in close proximity to another spider species, introduces potential for microbial exchange, either environmentally or through direct contact (horizontal or vertical transmission). Their web-sharing behaviour and small body size add a novel comparative dimension to this study, particularly in examining host–microbiome interactions in more socially or spatially connected environments.

Collectively, these species represent a wide and diverse ecological and behavioural spectrum, from the synanthropic *Pholcus* to the orb-weaving *Nephila* and kleptoparasitic *Argyrodes.* Subsequently, these three species offer an opportunity to explore how such differences shape microbiome composition. The next section presents the research aims and objectives that build on this comparative framework.

Research Aims and Objectives

This study aims to characterise and compare the prokaryotic microbiomes of two ecologically and phylogenetically distinct spider species, *Nephila senegalensis* and *Argyrodes argyrodes*, using 16S rRNA metabarcoding sequencing of arachnid tissue samples. In *Pholcus phalangioides*, DNA concentration was assessed from the quality control (QC) report generated during 16S rRNA metabarcoding of leg, abdomen, and head tissue samples; however, the full microbiome dataset was not captured. In contrast, for *Nephila* and *Argyrodes*, whole-body specimens were used to perform complete microbiome characterisation using metabarcoding. The experimental design included sampling *Pholcus* individuals across sex (male and female) and developmental stage (juvenile, subadult, and adult) and, in a small subset, individuals exhibiting the distinctive “whirling” behaviour, enabling interspecific and intraspecific comparisons of microbiome variation.

The specific objectives were:

- To examine and quantify the feasibility of sequencing the bacterial 16S rRNA genes of *Pholcus phalangioides*, using high-throughput metabarcoding.
- To characterise and compare the prokaryotic microbiomes of *Nephila senegalensis* and *Argyrodes argyrodes* using 16S rRNA sequencing.
- To describe interspecific and intraspecific variation in microbiome composition.
- To assess the presence and relative abundance of known endosymbionts (e.g., *Rickettsia, Wolbachia*) across individuals and species.
- To provide a baseline for understanding microbial diversity in arachnids.

This study emphasises quantifying, if feasible, the microbiome of *Pholcus phalangioides* as a first step in understanding the species' microbial community. By comparing the taxa identified here to microbes previously documented in other biological hosts, this work aims to establish a baseline for future novel ecological or functional analyses of arachnids and invertebrates alike. Where possible, it also explores potential associations between microbial presence and behaviour, offering initial insights into microbiome variation and host-microbe interactions.

Chapter 2 : Methods

Internal experiments: *Pholcus*, *Nephila and Argyrodes* samples

The *Argyrodes Argyrodes* samples were obtained from the Province of Cádiz, southern Spain, and the *Nephila senegalensis* samples were collected from KwaZulu-Natal, South Africa. One full-body sample of *Nephila and* two full-body samples of *Argyrodes* were sequenced from previously prepared and frozen DNA extracts, while the researcher collected 21 *Pholcus* individuals. All *Pholcus* individuals were collected in or around Nottinghamshire, East Midlands, UK, between autumn 2023 and summer 2024. Collection sites included the Life Sciences Building at the University of Nottingham, University Park campus (e.g., corridors, foyer), as well as private properties such as domestic dwellings and a local hair salon, all located in Hucknall, Nottinghamshire, UK. Specimens were captured using sterile specimen tubes.

All eight legs were dissected from the body of the spider, and DNA extraction was performed on one of the leg tissues of sample P1, belonging to a *Pholcus* individual. To maximise DNA yield, a SigmaAldrich GenElute™ Mammalian Genomic DNA Miniprep Kit was used, following the manufacturer’s protocol with two wash steps and no RNase treatment. DNA was eluted into nuclease-free water and stored at –20 °C. A negative extraction control of deionised water was included. A MPLEN nano volume nanophotometer, model N5, was used to quantify how much DNA was present in the P1 sample in group one. Groups were allocated to two groups of samples, group one contained one *Pholcus* individual (P1) and two *Argyrodes* samples (A9 & A17) and one *Nephila sample* (N). After internal DNA extractions, PCR (polymerase chain reaction) and gel electrophoresis had been conducted, the four samples (P1, A9, A17, and N) were exported to Macrogen Europe on standard ice packs. The

second group, group two, was composed exclusively of ten *Pholcus* individuals, containing twenty different types of tissue specimens that were shipped on dry ice (Table 2).

DNA extraction, polymerase chain reaction and sequencing

DNA was extracted from one *Pholcus phalangioides* individual (sample P1, group one) using a single leg specimen internally at the Spider Lab. To maximise DNA yield, a Sigma-Aldrich GenElute™ Mammalian Genomic DNA Miniprep Kit was used, following the manufacturer's protocol with two wash steps and no RNase treatment to maximise DNA yield. DNA was eluted into nuclease-free water and stored at –20 °C. A negative extraction control (deionised water) was included to identify any potential contaminants. To quantify the yield of DNA extraction and examine the concentration of the DNA in the sample, a microvolume UV-Vis spectrophotometer, Implen NanoPhotometer®, N50, was used.

Samples were tested using PCR amplification, which was conducted using a Techne Thermo cycler an initial denaturation at 94 °C for 1 min was followed by 35 cycles of 94°C for 30 s, 55 °C for 20 s and 72 °C for 30 s, using primers targeting arachnid host DNA, specifically the COI gene (Cytochrome c Oxidase Subunit I). This primer was originally described by Hedin and Maddison (2001), Hedin-O, and Hedin-C. The full primer sequences that were targeted are shown below in Table 1.

PCR amplifications were performed using Bioline MyTaq™ Red Mix (Bioline, UK), which contains Taq DNA polymerase, dNTPs, reaction buffer, and a tracking dye. Each reaction had a final volume of 5µL, consisting of 12.5µL Bioline ReadyMix, 0.4µM of each primer (from 10µM stocks), 2.5mM $MgCl_2$ (final concentration), 2µL DNA template, and nuclease-free water to volume. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 1 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s. Gel electrophoresis was performed using 1.5% agarose gels in 0.5× TBE buffer, stained with ethidium bromide, and visualised with the BioRad ChemiDoc Touch Imaging System. A 50 bp DNA ladder was used for size estimation.

Table 1 - List of primers and the accompanying genetic sequences 5' – 3' with their associated target regions utilised for internal PCR analysis.

Primer Type	Sequence 5' – 3'	Primer Target
Hedin-O	GAGAGAGTTCAAAGTCT	Host COI
Hedin-C	CGAGGTGAACGAGTGAT	Host COI

External: Macrogen

For the remaining twenty *Pholcus* tissue samples, an experimental pipeline at Macrogen South Korea was initiated, and all samples were subject to standardised DNA extraction and

metabarcoding 16S rRNA sequencing on dissected tissues, including the head, abdomen, and legs. No full-body *Pholcus* samples were used. Each *Pholcus* specimen was assessed for sex (male or female), developmental stage (juvenile, subadult, adult, or undetermined before being admitted to freezer storage (-18℃). The second group of samples, including the 20 *Pholcus* tissue samples, were shipped separately on dry ice. This sample set remained under the same conditions as those previously analysed in-house. All samples submitted to Macrogen underwent standard DNA extractions and subsequent 16S rRNA gene amplicon sequencing using a standard non-custom meta-amplicon package. The V3–V4 region of the 16S gene was amplified and sequenced on an Illumina MiSeq platform, generating 2 × 300 bp paired-end reads.

Data analysis

Raw 16S rRNA sequencing data were analysed in RStudio using the DADA2 pipeline using the following parameters: (maxN 0, maxEE c(2, 2), truncQ 2, rm.phix TRUE, compress TRUE and multithread FALSE). The DADA2 pipeline was utilised for the purposes of quality filtering, denoising, and pairing forward and reverse reads. Error models were then learned, and amplicon sequence variant inference was conducted. Forward and reverse reads were merged using the mergePairs() function, error models were learned with learnErrors(), and amplicon sequence variant (ASV) inference was conducted using dada(). Paired-end reads were trimmed and filtered based on quality scores using the plotQualityProfile() function. When default trimming parameters (DADA2 default: 0) resulted in poor overlap and some reads failed to pair, adjustments were made c(240, 160) to preserve read length and ensure successful sequence pairing. Additionally, chimeric sequences were identified and removed using the removeBimeraDenovo() function in the DADA2 workflow.

Taxonomic assignment was performed using the SILVA reference database (silva_nr99_v138.2_Genus), with ASVs assigned to the lowest possible taxonomic rank based on a minimum confidence threshold (MinBoot: 50). Sequences associated with mitochondria, chloroplasts, or unclassified domains were removed prior to downstream analyses. To maintain consistency and accuracy across samples, all putative eukaryotic sequences were excluded from downstream diversity metrics and composition analysis. Phylogenetic trees were constructed using the ggtree package in R Studio to visualise three distinct clades of *Serratia*. These overrepresented *Serratia* clades were subsequently excluded from further analysis to avoid skewing diversity metrics. ASV tables were imported into the phyloseq package for microbiome characterisation and visualisation. Alpha diversity was assessed using observed richness, Shannon index, and Simpson diversity index. Beta diversity was analysed using Bray–Curtis dissimilarity and visualised through Principal Coordinates Analysis (PCoA). Additional community composition analyses were supported using phyloseq functions, and, where applicable, the vegan package was utilised for multivariate statistics.

Preliminary Endosymbiont Screening Methods for *Pholcus* (2023)

DNA extraction

Genomic DNA was extracted using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's protocol, omitting the optional elution step to maximise DNA yield. DNA concentration and quality were assessed spectrophotometrically, and aliquots were stored at –18 °C.

PCR amplification

Polymerase chain reaction (PCR) was used to amplify target regions of *Wolbachia*, *Rickettsia* and host ribosomal RNA genes following published protocols (Majerus et al., 2000; Zchori-Fein & Perlman, 2004). Reactions (25 µL total volume) contained 1 µL of each 10 mM primer, 7.5 µL $MgCl_2$ (1.5 mM final), 7.5 µL H_2O, and 1 µL of DNA template. Thermal cycling was performed on a TechNET C-512 thermocycler with an initial denaturation at 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s; and a final extension at 72 °C for 5 min.

Table 2 – Primer names and associated sequences used to determine if endosymbionts (*Rickettsia and Wolbachia)* were present in *Pholcus*

Primer type	Sequence 5' – 3'	Primer target
WSP-F	TGGTCCAATAAGTGATGAAGAAACTAGCTA	*Wolbachia*
WSP-R	AAAAATTAAACGCTACTCCAGCTTCTGCAC	*Wolbachia*
RICS741-F	CATCCGGAGCTAATGGTTTTGC	*Rickettsia*
RICS1197-R	CATTTCTTTCCATTGTGCCATC	*Rickettsia*

Gel Electrophoresis

Amplified products were resolved by electrophoresis on 1.5 % agarose gels prepared in 0.5× TBE buffer containing 3 µL Ethidium Bromide per 100 mL gel volume. Entire 25 µL PCR reactions were loaded, and bands were visualised under UV illumination. The presence of an amplicon of the expected size was recorded as a positive infection.

Data handling & analysis

Infection status (positive (+)/negative (-) and behavioural category (whirler / non-whirler) were compared descriptively. Statistical significance was assessed using Fisher's exact test and the sign test, with $\alpha = 0.05$.

Preliminary Behavioural Observations of *Pholcus* (2024/25)

Behavioural data were informally recorded during *Pholcus*-specific specimen collection only. All live individuals were lightly stimulated using a fingertip to identify individuals exhibiting the characteristic "whirling" behaviour. Following behavioural observations and specimen collection, each specimen was humanely euthanised and later dissected into separate tissue types (head, abdomen, and legs). The specimens were then frozen at –18 °C or below; no alcohol was used to preserve the specimens.

Chapter 3: Results

Preliminary Behavioural and Endosymbiont Results for *Pholcus* (2023)

During a 2023 undergraduate project, seven *Pholcus Phalangioides* specimens were collected and assessed for the presence or absence of endosymbionts *Rickettsia* and *Wolbachia*; they were also simultaneously tested to determine if they demonstrated the *Pholcus*-specific whirling behaviour. PCR amplification followed by gel electrophoresis confirmed that some *Pholcus* do carry both *Rickettsia* and *Wolbachia*, whilst some spiders in the cohort did not test positive for any endosymbionts. The results confirmed that three spiders tested positive for endosymbionts: *Rickettsia* (2) and *Wolbachia* (1). The remaining four spiders did not test positive for either endosymbiont. Despite the positive bands for *Rickettsia* being faint on gel electrophoresis images, the resolution was considered high enough to be a positive result (Figure 1).

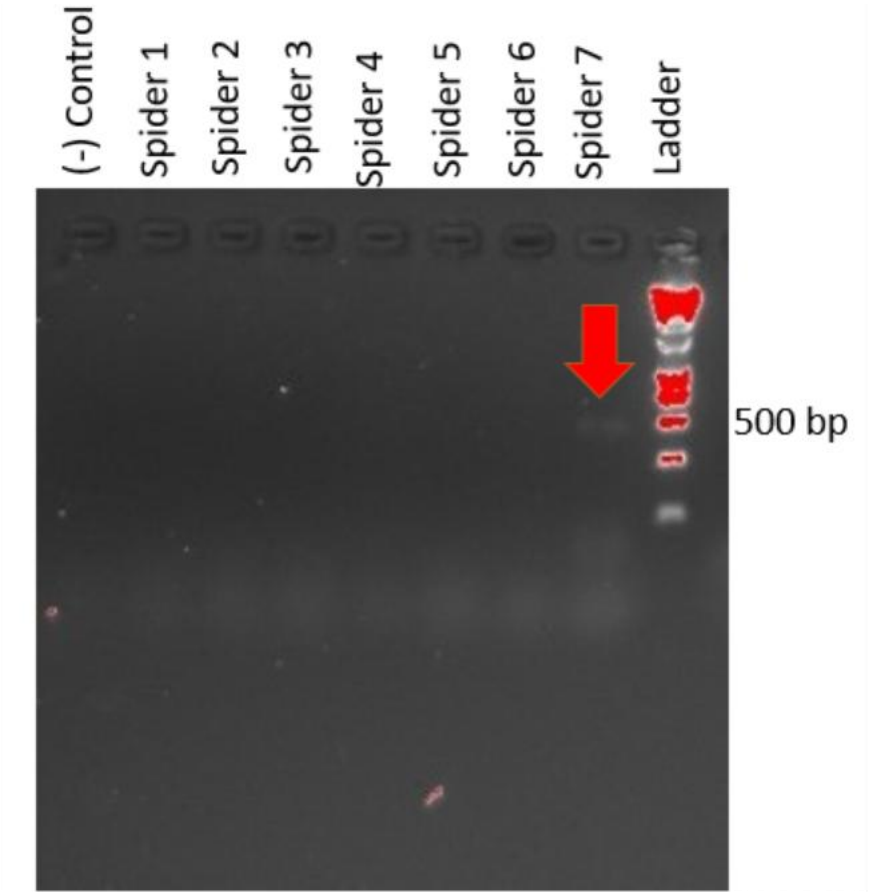

Figure 1 - Gel electrophoresis bands which visualise three positive endosymbiont results, two positive bands for Rickettsia, the most visible band being in spider 7. All three of these spiders were non-whirlers.

All three endosymbiont-infected individuals were non-whirlers, showing no response to fingertip stimulation. In contrast, all four uninfected individuals exhibited whirling behaviour when stimulated. Collectively, the behavioural assessments of this small cohort of *Pholcus* indicate that a weak or preliminary association was observed, which is demonstrated in Figure 2.

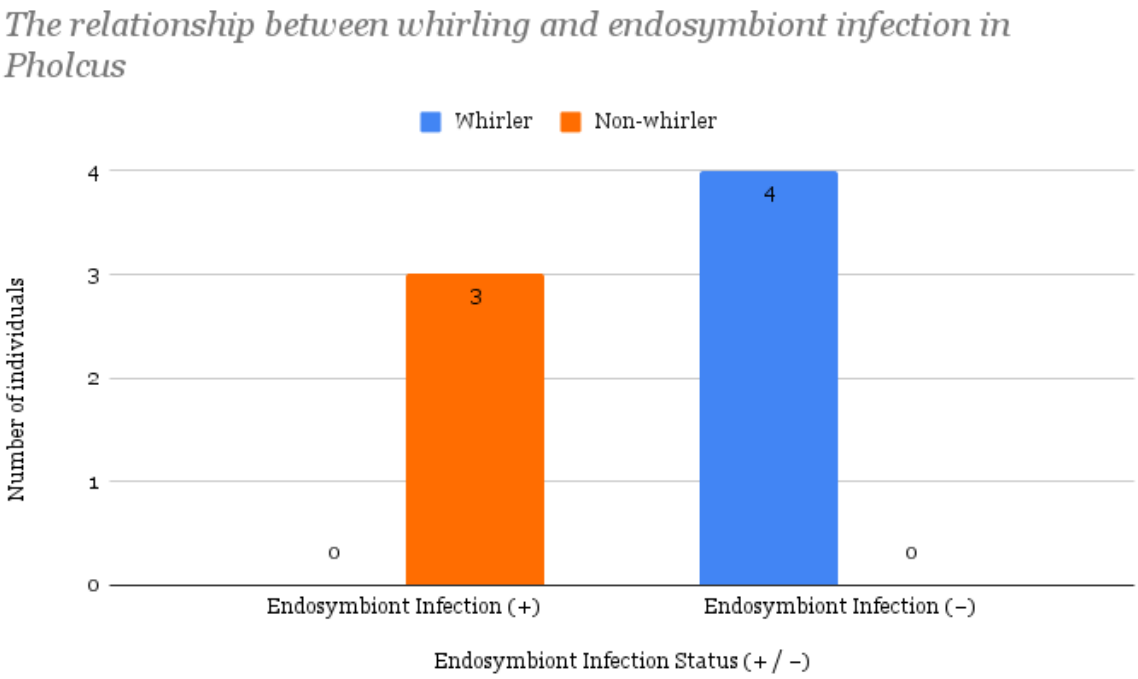

Figure 2 - Relationship between whirling behaviour and endosymbiont infection in *Pholcus phalangioides*. Bars represent the number of individuals with and without infection. Blue bars denote whirlers (behaviour +), orange bars denote non-whirlers (behaviour –).

Initial feasibility testing of sequencing for *Pholcus phalangioides*

This study aimed to characterise and compare the prokaryotic microbiome across two arachnid species: *Nephila senegalensis* and *Argyrodes argyrodes,* with quantitative comparisons being drawn from *P. Phalangioides (Pholcus phalangioides).* The preliminary results presented here are from DNA aliquots from *Nephila* and *Argyrodes* that underwent successful rRNA 16S metabarcoding sequencing, and a single *Pholcus* individual (P1) that underwent internal (Spider Lab) DNA extraction and PCR verification, as demonstrated in Figure 3.

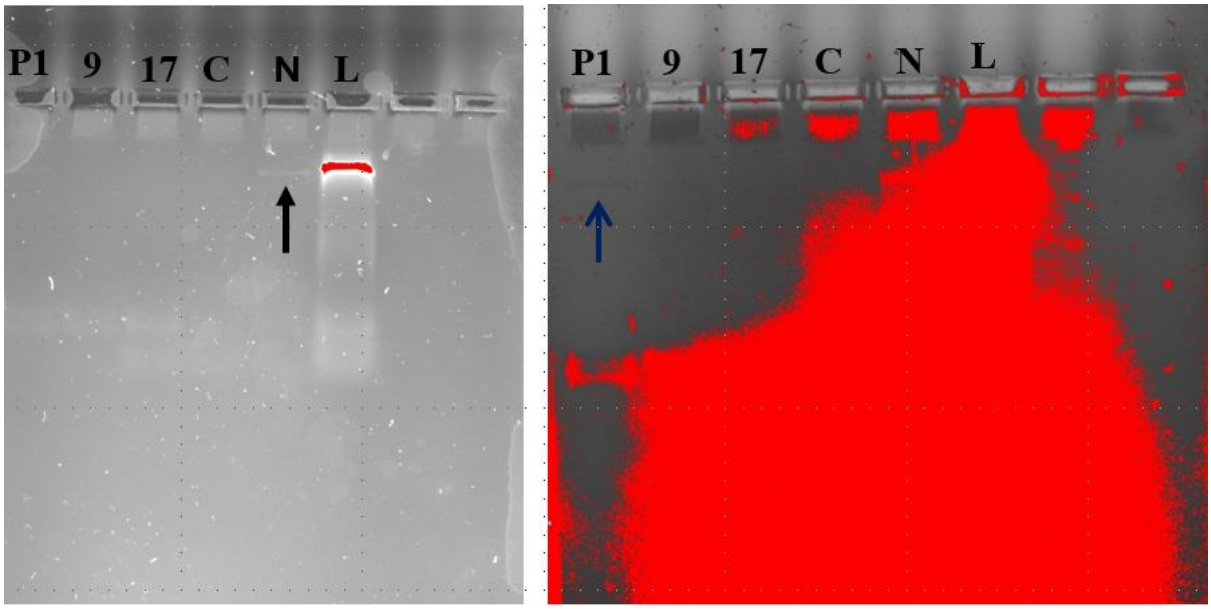

Figure 3 – Internal gel electrophoresis images that illustrate the positive band for arachnid host DNA in samples (P1 and N), Sample key: P1 = Pholcus, 9 = A9, 17 = A17, N = Nephila. C = negative control and L = ladder. The left image represents a lower UV exposure (approx ~2–3 mW/cm²), whilst the image on the right represents a higher UV exposure. (approx. ~6–8 mW/cm²).

However, the sample did not uphold expected quality control thresholds and did not yield any sequencing reads from external Macrogen sequencing. When the PCR products of sample P1 were run on gel electrophoresis, a faint positive band verified the successful amplification of arachnid DNA (Figure 3). However, 16S rRNA sequencing conducted externally (Macrogen, South Korea) did not meet the sequencing quality control (QC) thresholds. Thus, the P1 sample taken from group one was excluded from composition analysis.

Observed Whirling Behaviour in *Pholcus* (2024/25)

Behavioural observations were limited to *Pholcus* specimens and are presented here as preliminary data. Specimens were collected and tested between 2024 and 2025. Of the two individuals examined, one exhibited the characteristic whirling response following light fingertip stimulation, whereas the other did not (Table 3). The data presented in Table 3 follow directly from the preliminary behavioural data collected in autumn 2023 (see Section 3.1), as described by Goodacre and Clifton (2023, unpublished).

Table 3 - Two *Pholcus* individuals were assessed for whirling behaviour upon light tactile stimulation. A "+" indicates a whirling response; "–" indicates no observable whirling.

Spider specimen name	**Whirl status (+ or -)**
5: male, leg & head specimens	Whirler **(+)**
6: female, legs, abdomen & head	Non whirler **(-)**

Quantitative sequencing output for *Pholcus phalangioides*

Whilst this study did not characterise the microbiome of *Pholcus phalangioides* using downstream taxonomic assignment and diversity metrics, as it did for *Argyrodes* and *Nephila,* it still provides a quantitative foundational look at the feasibility of sequencing from a novel and previously uncharacterised organism, exemplified by the quantity of DNA and number of sequencing reads produced from each sample (Table 4).

Table 4 – DNA quantitative output of metabarcoding sequencing from sixteen *Pholcus* individuals.

Sample ID	Total Bases (bp)	Total Reads	GC%	AT%	Q20%	Q30 %	Tissue Type	Sex / Developmental Stage
10ML	49081060	163060	54.1	45.9	90.3	79.8	Legs	Male
1JL	52010392	172792	52	48	91.6	81.5	Legs	Juvenile (Unknown sex)
2A	74476430	247430	51	49	92.2	82.8	Abdomen	Female
2FL	61634566	204766	51.5	48.5	92.1	82.6	Legs	Female
3AF	83175932	276332	52	48	92.2	82.7	Abdomen	Female
3LFA	28985096	96296	52	48	91.2	81	Legs	Female
4HM	77952980	258980	51.1	48.9	92	82.5	Head & Abdomen	Male
4LM	68979568	229168	52.6	47.4	91.6	81.7	Legs	Male
5ML	63071540	209540	53	47	90.5	80	Legs	Male
6AF	68582248	227848	49.7	50.3	92.6	83.1	Abdomen & Head	Female
6LF	58798544	195344	50.7	49.3	91.9	82.1	Legs	Female
7WJ	67693094	224894	51.9	48.1	92.3	82.9	Whole body	Juvenile
8JA	78039668	259268	51.3	48.7	92.4	83.1	Abdomen & Head	Juvenile
8WL	57899156	192356	51.9	48.1	91.8	82	Legs	Juvenile
9FA	68575626	227826	51.8	48.2	92	82.4	Abdomen & Head	Female
9FL	65625224	218020	53	47	90.1	79.4	Legs	Female

In addition to this, quantitative comparisons were drawn from two groups: (tissue type and sex), and the total number of reads that were yielded from *Pholcus* individuals is shown in Figures 4. Figure 4 demonstrates the number of reads yielded from *Pholcus* individuals, and is plotted against the concentration of DNA that was extracted from *Pholcus* individuals during the external Macrogen pipeline. Figure 4 illustrates that the larger the quantity of DNA extracted from a sample, the more sequencing reads can be yielded from the specimens.

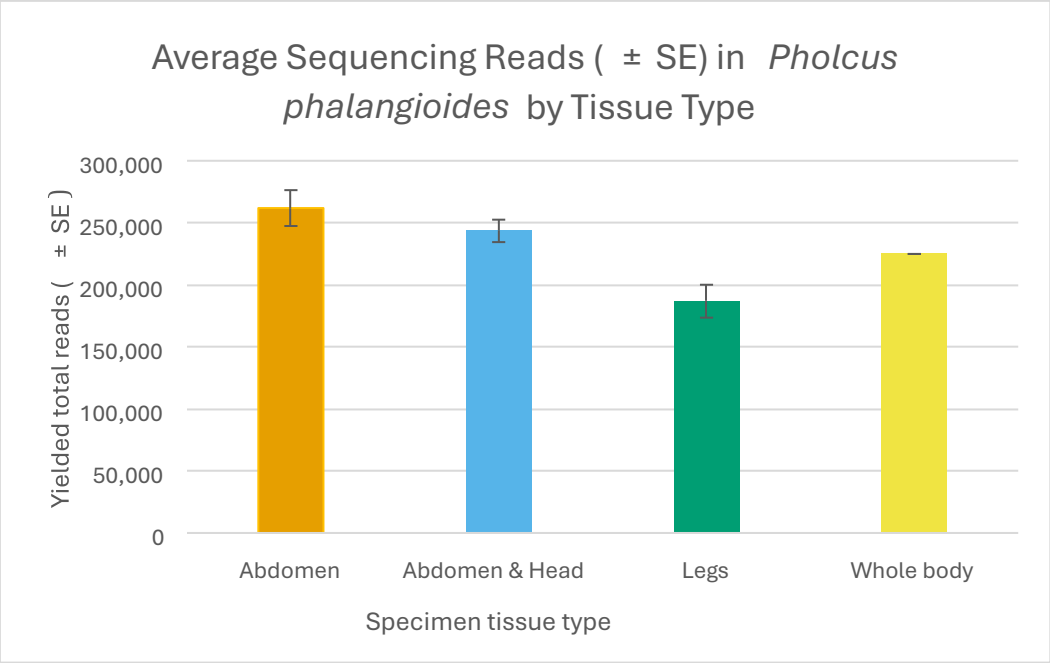

Figure 4 Average sequencing reads by tissue type

Figure 4 illustrates that the abdomens yielded the highest number of sequencing reads across specimen tissue types. The second tissue type to yield the most sequencing reads was the head and abdomen group, followed by whole-body specimens. The leg specimens yielded the least quantity of sequencing reads, with 185,000 metabarcoding sequencing reads, compared with the abdomen tissues that produced 250,000 reads. The error bars for Figure 4, which measure the amount of variation between replicates, demonstrate that when the bars are small, there is very little variation between replicates, which is true for the full-body *Pholcus* individuals. Therefore, highlighting that full-body specimens had almost identical sequencing reads produced across all replicates, which illustrates that sequencing was highly consistent.

In contrast to the leg specimens, which show the highest degree of inconsistent sequencing reads, signified by the larger error bars (Figure 4), demonstrating more variation amongst replicates and collectively, less reliability of the reads produced. Despite this, the remainder of the specimen groups examined (abdomen and abdomen & head) were somewhere in between the head and abdomen group; they demonstrated less consistency and more variation amongst sequencing reads and a larger error bar, suggesting that the reliability of these reads, if utilised in downstream analysis and taxonomic assignment, would not be optimal.

In addition to comparing the differences between tissue types, the sex of the *Pholcus* individuals was also compared with the quantity of total yielded reads. As highlighted by Figure 5, the sequencing reads yielded by *Pholcus* specimens of different sexes, including unidentifiable sex (juveniles), did not vary significantly in the average number of metabarcoding reads produced. Only minor variation was detected between males and juveniles, with female reads remaining generally consistent. As Figure 5 exemplifies, the female replicates within the dataset have the lowest error bars, and as a result, the yielded sequencing reads in female *Pholcus* will be the most reliable for downstream analysis and further characterisation, due to the increased consistency of reads sequenced. The same cannot be said for the male and juvenile groups, which have more prominent error bars, subsequently showing that the male and juvenile reads would have decreased reliability due to variability in the sequencing reads produced.

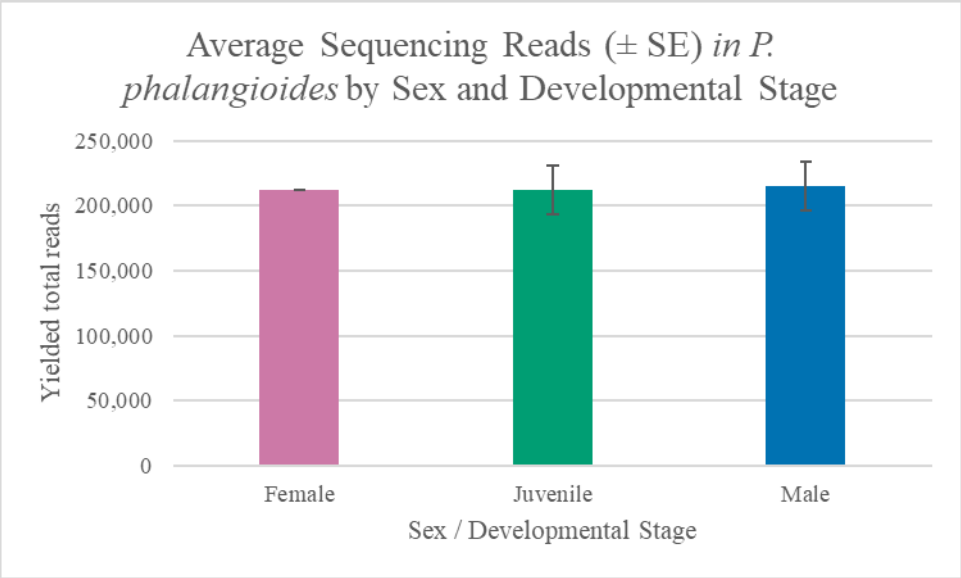

Figure 5 – Average sequencing reads by sex and developmental stage

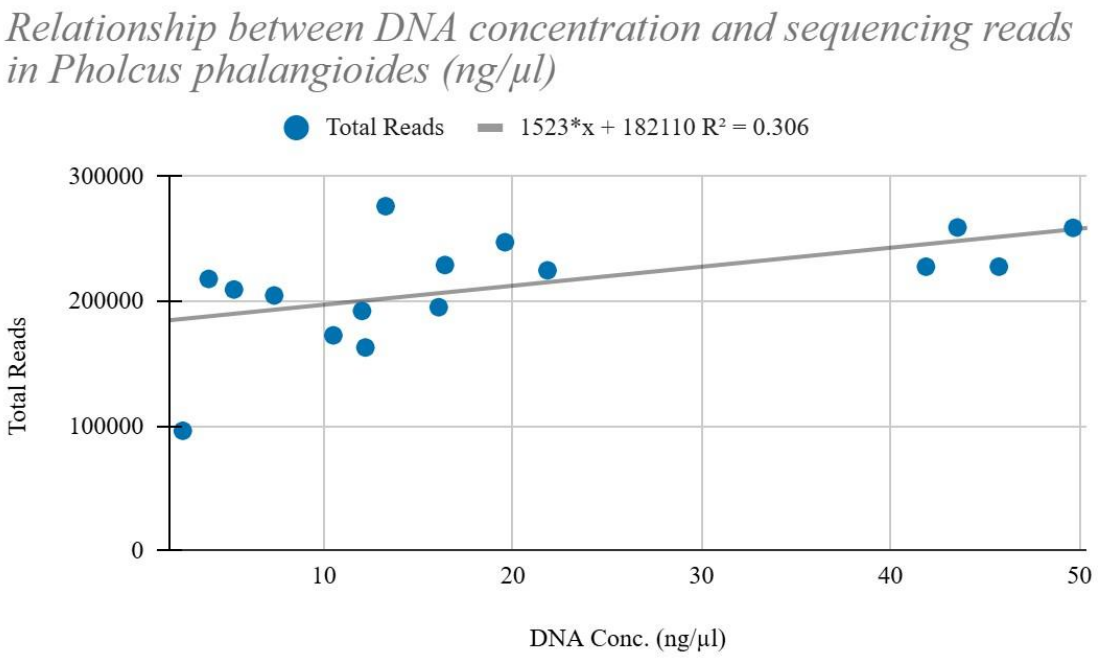

Figure 6 – Sequencing reads by DNA concentration

Microbiome Composition of *Argyrodes* and *Nephila*

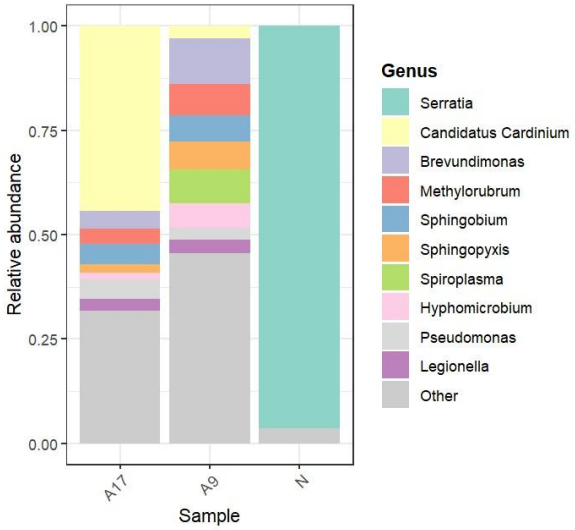

Figure 7 – Microbiome composition of Argyrodes and Nephila (A9, A17 & N) with Serratia dominance

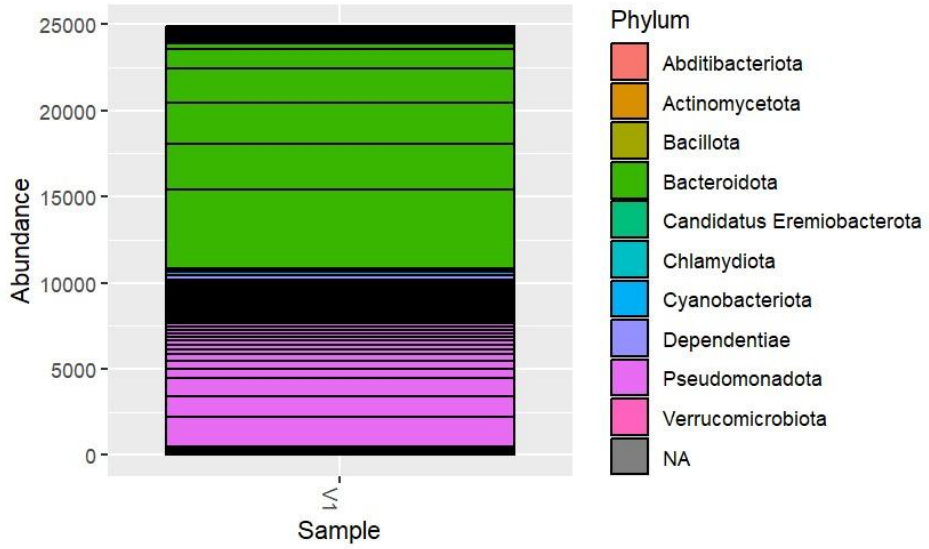

Figure 8 – Argyrodes (A17) microbiome composition.

The two *Argyrodes* individuals (A9 & A17) sampled in this study were sequenced and analysed. The stacked bar plot (Figure 8) shows phylum-level relative abundance for sample A17. This sample's microbiome was dominated by members of the phyla *Pseudomonadota, Bacteroidota,* and *Bacillota.* Minor contributions from *Verrucomicrobiota, Actinomycetota*, and *Cyanobacteriota* were also observed. Several reads remained unclassified at the phylum level, represented as "NA" (Figure 8). The Alpha diversity was evaluated using the Shannon diversity index, which accounted for both richness and evenness. The *Argyrodes* sample (A17) exhibited a Shannon index of approximately ~ 3.32.

Genus-level analysis of *Argyrodes,* sample A17, revealed that the microbiome is heavily dominated by *Candidatus Cardinium*; other genera detected at lower relative abundance include *Sphingobium, Methylorubrum, Pseudomonas,* and *Brevundimonas* (Figure 7). In contrast, the second *Argyrodes* individual (A9) exhibited a more taxonomically even and compositionally diverse microbiome. No single genus dominated, and a broader range of low to mid-abundance taxa were observed. Notably, none of the previously filtered *Serratia* ASVs were detected in this sample.

Intraspecific microbiome variation: *Argyrodes* samples

Shannon diversity indices were calculated for all three sequenced samples: *Argyrodes* A9, *Argyrodes* A17, and *Nephila* (N). Direct comparison of the two *Argyrodes* individuals revealed marked variation in microbiome composition. Sample A9 exhibited a Shannon index of 3.4, while A17 was lower at 2.6. The reduced diversity in A17 reflects the specimens' domination by the intracellular endosymbiont *Candidatus Cardinium* (Figure 7). Although both individuals carried *Cardinium* (A9 and A17), the disparity in relative abundance between them (Figure 7) suggests this symbiont may actively suppress microbiome diversity in certain hosts, driving microbiome homogenisation. This observation may point to individual or context-

specific differences in symbiont colonisation dynamics or competitive exclusion of other taxa.

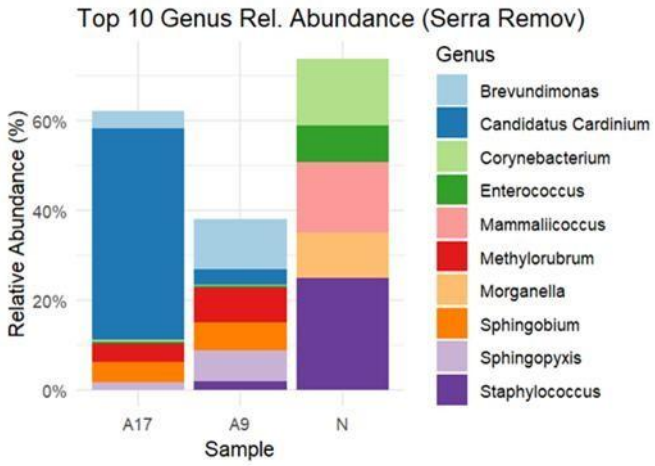

Figure 9 – Relative abundance of genus for Argyrodes and Nephila (A9, A17 & N) Serratia removed.

Moreover, rarefaction curves were used to assess the sequencing depth and genus richness across samples. Sample (A9) plateaued at approximately 65–70 genera, (A17) at 55–60, and *Nephila (N)* at only 10–15 genera. The *Nephila* curve saturated early (below 500 reads), as seen in Figure 10. This further supports that the species had reduced taxonomic complexity, even following the removal of dominant *Serratia* amplicon sequencing variants (ASVs).

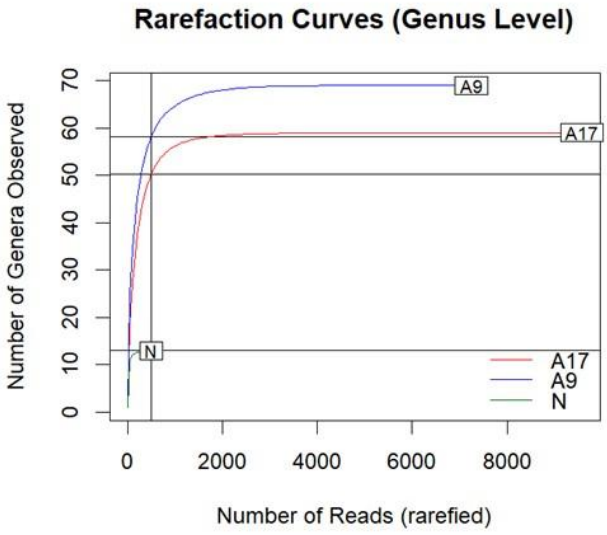

Figure 10 - Rarefaction curve (genus-level richness) in Argyrodes and Nephila (A9, A17 & N).

Although the *Nephila* sample (N) was initially excluded from diversity analysis due to overwhelming *Serratia* dominance (Figure 7), further breakdown of the remaining taxonomic profile (Figure 9) revealed several noteworthy genera. Among the top "other" genera detected were *Staphylococcus, Mammaliicoccus, Corynebacterium,*

and *Morganella—Staphylococcus* alone accounted for nearly 25% of the filtered community.

Furthermore, statistical analysis conducted in R Studio examined the phylogenetic relationship between the endosymbiont *Serratia* and the arachnid host, *Nephila*. When the sequencing reads of all known strains of *Serratia* were compared using BLAST, it became clear that not only one clade of *Serratia* was present in a single arachnid host, but three clades that were phylogenetically distinct. The sequencing depth of each sample is represented by the size of the circles on the figure (Figure 11).

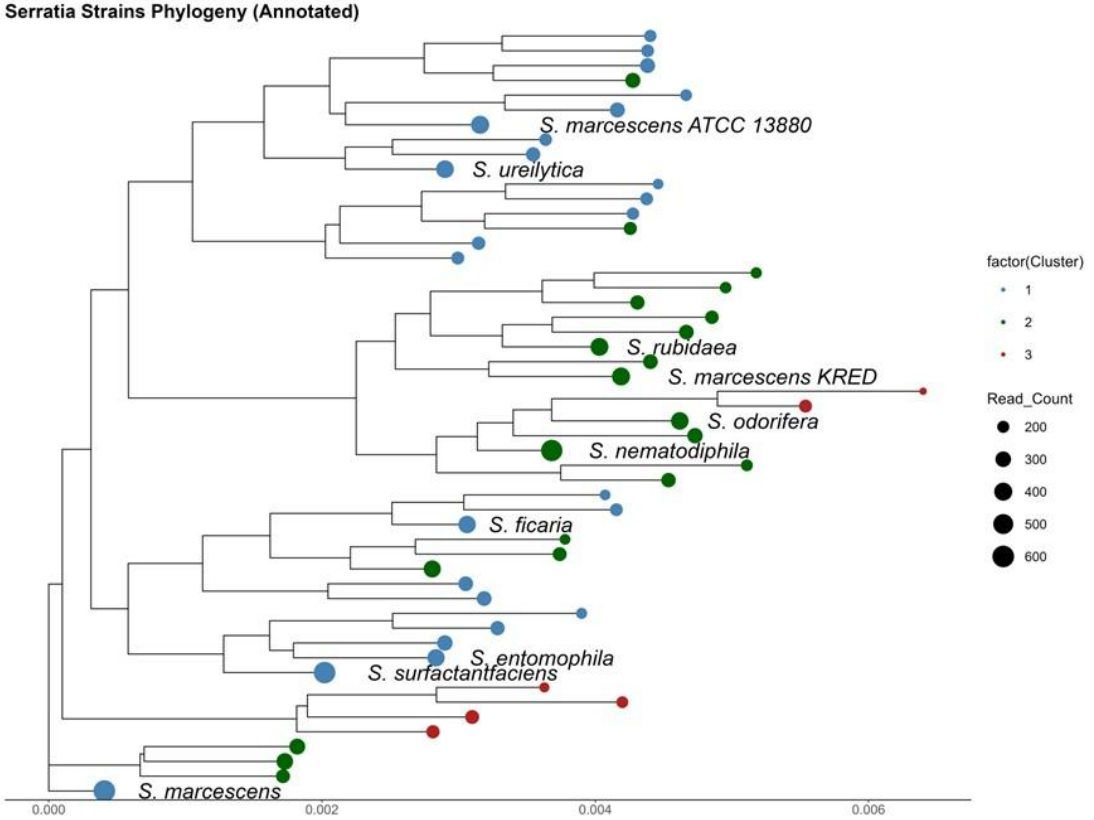

Figure 11 - Phylogenetic tree illustrating three clades of Serratia sequenced from Nephila (N).

Cluster one in the tree is defined by blue circles, cluster 2 is defined by green circles and cluster 3 is defined by red circles. This illustrates each strain within a clade; the further away an individual branch is from the common ancestor, the more genetically distinct they are and vice versa. Additionally, the tree explains the causation of evolutionary divergence, and another important point to make would be that all strains within a clade are very similar and therefore present no significant differences phylogenetically, which is shown by the length of the branches that extend from each individual strain on the tree.

The *Nephila* sample, consisting of a full-body DNA aliquot, exhibited a strong overrepresentation of the bacterial genus *Serratia* (Figure 7). Phylogenetic tree construction using the ggtree package in R revealed the presence of three distinct Serratia clades—ASV34, ASV42, and ASV52—Cluster 1, Clade 2, and Clade 3,

respectively (Figure 12). These variants represent a tight monophyletic group and are disproportionately abundant in the *Nephila* sample.

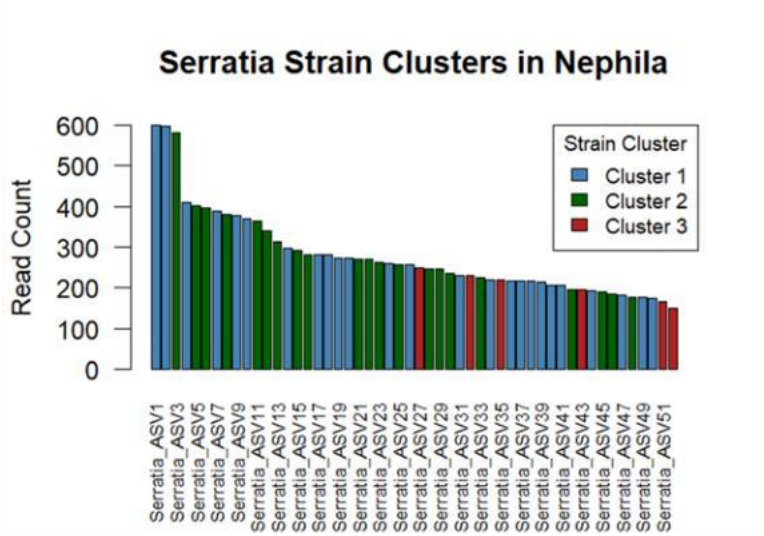

Figure 12 Read counts of individual Serratia amplicon sequence variants (ASV'S) detected across a single Nephila sample, grouped by strain cluster.

Summary of findings

The primary aim of this study was to novelly characterise the microbiome of *Pholcus phalangioides* . During the study, additional comparative samples from *Nephila senegalensis* and *Argyrodes Argyrodes* were incorporated to enable interspecific comparisons across three ecologically and phylogenetically distinct spider taxa. This expanded sampling set allowed for broader analysis and comparisons of arachnid microbiomes across different habitats, lifestyles, and geographical regions, facilitating the generation of richer metadata.

Chapter 4 : Discussion

Evidence for the absence of a core microbiome in some Arachnids

The findings of this study suggest that arachnids examined here do not demonstrate evidence of a traditional 'core' microbiome, which directly correlates with the literature that has already assessed this in arachnids, with some variation observed in invertebrates (Engel, Martinson and Moran, 2012; Vanthournout, Vandomme and Hendrickx, 2014). Interestingly, here, individuals of the same species do show similar intra-specific microbiome compositions (A9 & A17). In the case of *Argyrodes,* the microbiomes are not identical, and predominantly abundant taxa within the top ten are shown to be present in differing abundances – this could demonstrate a small, not entirely characterised microbiome in the species. Similarly to what was observed here in *Argyrodes*, Kumar et al. (2020) examined the intraspecific differences between individual spiders from the same environments. They found variability in the host

microbiome compositions, but no clear core microbiome was defined. They cited horizontal transmission and acquisition as the likely cause of any observed differences between individuals (Kumar et al., 2020).

Microbiome acquisition: horizontal vs. vertical transmission

The results of this work correlate with research (Dunaj et al., 2020; Rose et al., 2023), which shows that Arachnids primarily acquire their microbiomes from horizontal sources, including the extrinsic environment the spider interacts with and the food sources they consume (Ahmed et al., 2015). Spiders are less likely to inherit their microbiome maternally, unlike other invertebrates (Powell et al., 2014; Kwong and Moran, 2016; Wu et al., 2021). However, despite the lack of a core microbiome, most endosymbionts within arachnid and invertebrate hosts are vertically transmitted (Werren et al., 2008). Nonetheless, honey bees possess a core microbiome and still carry maternally inherited endosymbionts, whilst arachnids do not have a core microbiome but carry endosymbionts that are maternally transmitted. This comparison shows that the presence or absence of a core microbiome does not preclude the mode of inheritance utilised by endosymbionts. Further, this highlights that microbiome composition and inheritance of symbionts represent distinct and non-mutually exclusive processes.

Endosymbiont patterns: presence, absence, and abundance

A notable result of this study is that *Cardinium* was found in both full-body *Argyrodes* samples (A9 and A17) in varying abundance, aligning with previous research (Duron et al., 2008; Goodacre et al., 2006). No other endosymbionts were detected within the same samples, either interspecifically or intraspecifically, contrasting with previous studies that reported the presence of co-infections involving multiple endosymbionts in individual spiders (Duron et al., 2008; Goodacre et al., 2006; Curry et al., 2015).

Biological significance and artefactual origins of detected bacterial taxa

The bacterial communities characterised across *Argyrodes* and *Nephila* reveal a complex mixture of biologically significant symbionts and potential artefactual contaminants. At the phylum level (Figure 8), the *Argyrodes* sample (A17) was dominated by *Bacteroidota* and *Pseudomonadota*, phyla commonly associated with arthropod microbiomes (Engel and Moran, 2013). Additionally, *Candidatus Cardinium* was found in both *Argyrodes* and *Nephila* specimens, suggesting a genuine ecological association rather than contamination. *Cardinium* are intracellular endosymbionts similar to *Wolbachia* and *Rickettsia*, known for manipulating host reproduction through mechanisms such as cytoplasmic incompatibility (Zchori-Fein and Perlman, 2004).

In contrast, *Brevundimonas*, *Sphingobium*, and *Methylorubrum*—detected in both *Argyrodes* samples (A9 and A17)—are frequently reported as reagents or environmental contaminants (Salter et al., 2014). Similarly, *Cyanobacteria* are photosynthetic organisms often detected as

contaminants in metabarcoding studies because their DNA is common in laboratory reagents and dust, particularly in low-biomass extractions (Tanner et al., 1998; Salter et al., 2014). These taxa are therefore considered artefactual and unlikely to represent true biological or ecological associations within the host microbiome.

Despite this, the high abundance of *Enterococcus* observed in the *Nephila* sample (N) suggests biological significance, as species of this genus are known to initiate and modulate digestive processes in invertebrates (Lebreton, Willems and Gilmore, 2014). Likewise, the overdominance of *Serratia* is also likely to represent a meaningful host-associated taxon in *Nephila* given its diverse ecological functionality. *Serratia* species can occur as gut symbionts, opportunistic pathogens, or vertically transmitted endosymbionts. In aphids, *Serratia symbiotica* forms a well-characterised mutualistic relationship with its host, supplementing essential amino acids absent from the phloem-based diet (Burke et al., 2009). Consequently, the high abundance of *Serratia* detected here is more consistent with a stable, host-associated relationship than with laboratory or reagent contamination. However, the overwhelming dominance of *Serratia* can obscure other taxa and distort perceived community richness, necessitating filtered analyses for accurate interpretation.

Overall, these results highlight the importance of exercising caution when analysing low-biomass 16S rRNA microbiome datasets due to the inherent risk of reagent contamination, statistical noise, and dominance by particular taxa, all of which can distort diversity metrics. While several taxa in this dataset—such as *Cardinium*, *Enterococcus*, and *Serratia*—likely represent biologically significant associations, others are artefactual. Future work should incorporate no-template controls to better account for background microbial signals and to minimise contamination artefacts. Finally, the rich abundance of *Serratia* reads in the *Nephila* sample (N) appears biologically and functionally significant and warrants further investigation.

Serratia as a dominant and disruptive taxon

Similarly, the full-body *Nephila* sample (N) did not appear to have any endosymbiont infections present within its top ten genera, even after removal of the dominant bacteria *Serratia*. Whilst this does not mean endosymbionts were absent, it suggests that if they were present, they were not detected in high enough abundances to meet the minimum taxa assignment threshold. Hundertmark (2019) cited this as a common problem when sequencing and analysing 16S rRNA data, highlighting that filtering out low-abundance OTUS (operational taxonomic units) during data analysis pipelines can exclude endosymbionts from downstream analysis, even when they are seemingly abundant (Hundertmark, 2019). This could have been the cause of the apparent absence of endosymbionts in the *Nephila* sample presented here.

This implies that the microbiome composition of the *Nephila* sample could not be colonised by endosymbionts. A suggestion for why this result was observed would be that, due to the overarching dominance of *Serratia* in this sample, the overall

microbial diversity was reduced, with *Serratia* pushing the microbiome towards homogenisation. Additionally, *Serratia* could have caused some kind of physiological modulation that inhibited endosymbionts from colonising the host niche, as previously evidenced by Luo et al. (2021). The result observed in this study, which showed that sample N had a low overall microbial diversity with similar taxonomic composition, is supported by Lamelas et al.'s (2011) research that cites *Serratia* as a microbe known to cause dysbiosis and disrupt host symbiotic balance, especially during periods of stress or compromised immunity. The same study also noted that *Serratia* displaces and inhibits co-occurring microbes. In summary, if the results showing the absence of endosymbionts and coinfections are assumed to be reliable, it can be inferred that *Serratia* has either outcompeted the endosymbionts (*Wolbachia* or *Cardinium)* or modulated a physiological change within the host niche so that others cannot colonise. The *Argyrodes* samples (A9 and A17) were infected with *Cardinium*, but, contrastingly, these were not simultaneously infected with *Serratia,* suggesting that they share an antagonistic relationship.

Method limitations: laboratory

Despite successful DNA extractions, positive PCR results, and visible gel electrophoresis bands (under in-house laboratory conditions) for both arachnid and endosymbiont DNA, these findings do not correlate with the expected results of external 16S rRNA amplicon sequencing, as they did not meet the minimum threshold requirement for quality control testing. Reasons for this may have included the use of sub-optimum Taq Polymerase in the PCR amplification of host and endosymbiont DNA, decreased efficacy of primers, inadequate PCR conditions, the use of standard ice packs for international shipment, or opting not to use ethanol as a preservative to comply with international export policy.

One possible reason for the unsuccessful sequencing of sample P1 is the use of sub-optimum thermostable DNA polymerase (Taq Polymerase) during in-house PCR amplification. Although Taq is generally considered robust and maintains efficacy up to and beyond its expiry date when stored properly (Youngblom, 2003; Liu and LiCata, 2013). However, its performance can decline if repeatedly thawed and refrozen (Lopez et al., 2024). In this study, the enzyme was thawed only once and returned immediately to freezer storage following use, but the fact that it had previously been opened and reused raises the possibility of reduced efficacy. Furthermore, the reagents used to stabilise Taq may degrade over time, potentially compromising enzyme performance (Cieślińska et al., 2014).

The use of standard ice packs for international shipment can be effective for short-term DNA preservation, as demonstrated by three out of four samples sent to Macrogen South Korea: two *Argyrodes* and one *Nephila (*A9, A17, and N), all of which passed external quality control (QC) checks. However, sample P1 (*Pholcus*) failed to meet QC requirements. DNA degrades rapidly when exposed to elevated temperatures (Straube and Juen, 2013; Oosting et al., 2020), and the underestimation

of how quickly this can occur during international transit may explain the discrepancy between the positive in-house PCR results and the external QC failure within the Macrogen pipeline.

Notably, one sample that passed QC belonged to a larger-bodied spider species, *Nephila*, which can be up to ten times the size of *Pholcus*, while *Argyrodes* are roughly two to five times smaller than *Pholcus*. This size difference is biologically significant because a larger body mass typically yields a greater quantity of DNA (Miller et al., 2013). Therefore, even if some DNA degradation occurred in transit, the remaining quantity in the *Nephila* sample was likely to have been sufficient to meet QC thresholds. In contrast, *Pholcus* would be expected to yield a lower quantity of DNA; any further degradation during transport would make the sample less likely to pass QC.

The second group of *Pholcus* samples sent to Macrogen, South Korea were shipped on dry ice (as compared with the first sample set shipped on standard ice packs), with the aim of preserving DNA integrity and increasing the likelihood of obtaining high-quality reads for all samples, a practice commonly used during metabarcoding microbiome studies (Andersen et al., 2021; Sheerin, 2023). Unsurprisingly, the second group, which contained twenty *Pholcus* specimens, met the minimum QC requirement and were sent through the remainder of the Macrogen pipeline for metabarcoding sequencing. These findings indicate that the shipping conditions of the samples were the most likely cause of the P1 QC failure. Only a small sample size of each specimen was sent as a first batch trial. This was done purposefully to identify and resolve any potential issues with the external sequencing pipeline, while minimising delays and sample losses for the project overall.

Another factor that could have influenced the failure of P1 in the external sequencing pipeline was the decision not to use ethanol as a preservative for any of the specimens. Ethanol, being a flammable alcohol, is deemed an international shipping hazard, and as such, was excluded from the preservation protocol to avoid any delays associated with customs clearance. Furthermore, because freezing the samples was the primary method of euthanising and storing specimens, ethanol was additionally deemed unnecessary.

Cooper (2011) challenged this approach, arguing that euthanising arachnids is not the most humane practice and may diminish the preservation of tissue integrity, despite freezing being a commonly used methodology of euthanasia within laboratory settings (Bennie et al., 2012; Tucker et al., 2023). This supports the theory that the preservation techniques used for P1 may have contributed to the metabarcoding sequencing failure. As an alternative, Cooper (2011) suggests using a euthanasia technique of immersion in 70% ethanol for not only improved preservation of tissue integrity but, of the utmost importance, increased animal welfare.

The decision to exclude ethanol as a preservative might have been reconsidered under different circumstances, for example, if the samples were to be sequenced in-house or shipped within the UK, where fewer shipping restrictions apply. Moreover, there is evidence that storing samples in ethanol helps minimise DNA degradation during storage in both insects and arachnids (Moreau et al., 2013; Sudhikumar, 2015). In 2021, Marquina and colleagues proved that ethanol, particularly at high concentrations (≥95%), is effective in preserving DNA over time in insects. However, the study also noted that ethanol can cause specimens to become brittle and may compromise morphological integrity as samples become denatured and dehydrated due to ethanol exposure (Dawson, Raskoff and Jacobs, 1998; Marquina et al., 2021).

In contrast to the previous points, Sales et al. (2020) refute the hypothesis that either method of preserving an invertebrate specimen (storage in ethanol or freezing the samples) makes a difference when their study compared both methods against DNA quantity, purity and PCR amplifications in flies over a period of 12 months. Whilst this might be true for the sand flies examined in the study, it was not the result observed here, as all the arachnid samples were exported abroad and were exposed to uncontrolled extrinsic variables outside of a controlled sterile laboratory environment. Additionally, Sales (2020) suggested that the preservation method of choice should be guided by practicality and the working environment, rather than efficacy alone. In this time-sensitive case, freezing was the most convenient and logistically feasible option.

Furthermore, Dawson, Raskoff, and Jacobs (1998) examined the effectiveness of freezing specimens in preparation for DNA analysis. They found that freezing can be a reliable technique, but DNA integrity declines with time prior to processing after thawing. Additionally, repeated freeze–thaw cycles were identified as a key factor contributing to DNA degradation, which can negatively affect downstream analysis. Taken together, the combination of improved shipping conditions (e.g., dry ice) and the use of ethanol preservation could be beneficial for future studies involving similar organisms. This dual approach would also be worth adopting in replications of this study to increase successful metabarcoding sequencing during initial processing.

Another possible reason for the failure of metabarcoding sequencing in sample P1 was an unexpected result from the NanoDrop spectrophotometer (NanoDrop) used during initial DNA quantification. The NanoDrop indicated the presence of DNA in the blank control (deionised water) but failed to detect DNA in the arachnid sample. This anomaly was unexpected, as PCR and subsequent gel electrophoresis confirmed positive results for both arachnid and endosymbiont DNA, with no amplification visible in the control. These findings suggest a possible NanoDrop calibration error. Although sample mislabelling was initially suspected, this was later ruled out based on consistent PCR and gel electrophoresis outputs.

Simbolo et al. (2013) assessed spectrophotometric DNA quantification using NanoDrop. They noted that NanoDrop Spectrometers tend to be unreliable at low DNA concentrations (<10 ng/µL). However, the approximate concentration of DNA

contained within P1 was around (~20ng/µL), which should have been within the detectable range for the NanoDrop Spectrometer. Additional limitations of the instrument include sensitivity to residues, air bubbles, and sources of contamination in controls (GarcíaAlegría et al., 2020), which may have resulted in the anomalous result obtained when quantifying P1. Simbolo et al.'s (2013) study also outlined a recommendation to use Qubits over NanoDrops as a more reliable technique for quantification.

During taxonomic classification, several sequences from the *Argyrodes* dataset were assigned to eukaryotic taxa, despite the analysis targeting prokaryotic 16S rRNA genes. These reads likely reflect either off-target amplification, low-level host DNA contamination, or database misassignments. Importantly, the version of the SILVA database used in this analysis (silva_nr99_v138.2_Genus) is designed primarily for prokaryotic taxonomic resolution and does not reliably classify eukaryotic sequences, which limits the interpretation of the eukaryotic reads assigned taxonomy during downstream analysis.

Similarly, Allen et al. (2016) argue that metabarcoding sequencing can lack sufficient depth and sensitivity, leading to underestimations of microbial diversity and abundance. In addition, intraspecific variation in microbial abundance poses a further challenge in arachnid microbiome studies (Vanthournout and Hendrickx, 2015), as observed in this study, in the *Argyrodes* samples. The intraspecific variation in microbial abundance observed in arachnids, as described by Vanthournout and Hendrickx (2015), poses challenges for recognising consistent microbial patterns. Therefore, this natural variability can hinder efforts to define species-specific microbiomes.

Sample (N) was heavily dominated by the gram-negative bacteria *Serratia*, which prevented the entirety of the spider's microbiome from being characterised and statistically analysed. *Serratia* was not proven to be an overarching dominant Genus within the other two samples, *Argyrodes* (A9 & A17); in fact, it was absent from them altogether, further supporting the hypothesis that the *Serratia* clusters sequenced were sample-specific to *Nephila* and likely non-biological in origin. As a result, to prevent skewing of diversity metrics, all three *Serratia* ASV clusters were removed from downstream composition and diversity analyses, which allowed for all low to medium abundance taxa within that sample to be identified, characterised and included in the dataset. Although the *Serratia* ASVS had to be filtered out and removed from the R analysis pipeline, they were examined independently before removal to comprehend whether only a single strain of *Serratia* colonised the arachnid host. In this case, multiple *Serratia* strains from three different clades were present within one individual, Nephila (N).

Cardinium, a known intracellular endosymbiont in arthropods, was detected in both full-body *Argyrodes* samples (A9 and A17), but in differing abundances, suggesting intraspecific microbiome variation. This is notable given that the literature presents

conflicting evidence about whether endosymbionts—particularly *Cardinium*, *Rickettsia*, and *Wolbachia*—show significant variation across tissue types. For example, Sheffer et al. (2019) found no notable differences in bacterial communities across different tissues in the wasp spider *Argiope bruennichi*, indicating potential body-wide microbial uniformity. In contrast, the variation observed in this study may reflect individual-level differences rather than tissue-specific distribution. It is also important to acknowledge that the sampling size for both *Nephila* and *Argyrodes* was limited, as these species were included primarily for comparative purposes with *Pholcus*, the focal species of this study. While the dominance of *Cardinium* may suggest a symbiotic relationship in *Argyrodes*, additional sampling is needed to determine whether this pattern is consistent across individuals or populations.

Pholcus (whirling) behavioural metadata evaluation

Fingertip stimulation was used to assess whirling behaviour in all *Pholcus* individuals prior to euthanasia. Although behavioural metadata were recorded, they were not analysed in this study. This decision was informed by a previous undergraduate project (Goodacre and Clifton, 2023, unpublished), which found a weak negative correlation between whirling and endosymbiont infection in a small sample of seven individuals. In this work (Goodacre and Clifton, 2023, unpublished), gender was not recorded, and leg tissue specimens were used exclusively, meaning that no accurate metadata in relation to sex, developmental stage, or any other tissue types was determined. Additionally, the leg specimens used were not sequenced using metabarcoding, which significantly limited the scope of the study in terms of sequencing the full microbiome of an individual and producing subsequent diversity metrics and analyses.

Despite its limitations, the undergraduate project recorded the presence or absence of webs for each *Pholcus* individual, an environmental variable uncaptured in the current study. This information could have provided valuable context when comparing endosymbiont infection status with behavioural traits such as whirling. In the earlier work, three *Pholcus* individuals tested positive for endosymbionts and were classified as non-whirlers, while the four whirlers showed no detectable endosymbionts. These preliminary findings motivated the inclusion of behavioural observations in the present study.

However, due to the extremely limited number of observed whirling individuals, and because collection of specimens was carried out by different individuals under varied conditions, reliable and standardised metadata for behaviour could not be obtained. As a result, behavioural data were treated descriptively and excluded from statistical analyses and direct comparisons with microbiome sequencing results. Due to the presence of only a single *Nephila* individual in group one, no intraspecific comparison could be made for this species, and as a result, it has been excluded from diversity-based conclusions accordingly. Nonetheless, compositional insight from this sample remains relevant as an interspecific comparison between *Argyrodes* and *Pholcus,* which were utilised within this study and are discussed here.

Changes to original project scope: *Pholcus*

Despite the overall success of the study, the original scope of this work was to characterise the microbiomes of *Pholcus phalangioides*, *Argyrodes argyrodes*, and *Nephila senegalensis.* While metabarcoding sequencing proved straightforward for *Nephila* and *Argyrodes, Pholcus* presented challenges during quality control checks in the external sequencing pipelines. Due to project time constraints and the fact that the raw FASTQ files were only received during the thesis write-up, the *Pholcus* reads were not synthesised or analysed here, slightly altering the scope of the study.

Limited sample size for *Pholcus* and *Nephila*

Due to the presence of only a single *Nephila* individual in group one, no intraspecific comparison could be made for this species, and as a result, it has been excluded from diversity-based conclusions accordingly. Nonetheless, compositional insight from this sample (N) remains relevant as an interspecific comparison between *Argyrodes* and *Pholcus,* which were utilised within this study and are discussed here.

Chapter 5 : Conclusion

This study quantified the amount of microbial DNA yielded from a previously uninvestigated arachnid, *Pholcus phalangioides*, using high-throughput 16S metabarcoding sequencing. The prokaryotic microbiomes of *Nephila senegalensis* and *Argyrodes argyrodes* were examined and characterised. The research assessed and demonstrated the feasibility of sequencing the microbiome of *Pholcus phalangioides.* The microbiomes of *Nephila* and *Argyrodes* were then compared, allowing diversity metrics of ecologically and phylogenetically distinct spider species to be examined.

The inability to analyse and characterise the *Pholcus* metabarcoding sequencing reads means that this study could not determine if the key findings for *Argyrodes* and *Nephila* are true for *Pholcus* as well. For example, does *Pholcus* have a core microbiome? Is there any intraspecific diversity observed between individuals? This is important because it outlines the composition of the microbiomes of samples: (A9, A17 and N) in the present day. Establishing this baseline enables microbiome comparisons to be drawn over evolutionary time. This metabarcoding sequencing data may contribute to future research, particularly those centred around evolution or conservation.

The study also found intraspecific microbiome differences among *Argyrodes* individuals, evidenced by variable *Cardinium* abundances observed in two individuals, which might be part of a small core microbiome in this species. Furthermore, there were interspecific differences between the microbiomes of *Argyrodes* and *Nephila*, likely due to spider lifestyle, ecology, and phylogenetics. This research has evidenced the successful microbiome sequencing of arachnids.

This work demonstrates that the methodology developed to yield microbial DNA and sequencing reads is likely applicable to other small invertebrates, potentially enabling further analysis in the form of characterisation using taxonomic assignment and diversity metrics. This research introduces the potential for more detailed comparative studies of arachnids and invertebrates alike, both intraspecifically and interspecifically. Additionally, this study contributes to knowledge surrounding sequencing methodology, host ecology, host–microbe interactions, evolution, and symbiotic relationships between hosts and endosymbionts. In doing so, the study lays fundamental groundwork for designing and experimenting on novel invertebrate microbiomes and provides foundational knowledge of arachnid microbiomes, their similarities and differences, and the factors that can influence or modulate their composition.

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