UNIVERSITÉ DU QUÉBEC À MONTRÉAL

THE GUT MICROBIOTA'S TEMPORAL DYNAMICS AND RELATIONSHIPS WITH INDIVIDUAL TRAITS IN A WILD EASTERN CHIPMUNK POPULATION

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RELATIONS ENTRE LA DYNAMIQUE TEMPORELLE DU MICROBIOTE INTESTINAL ET LES TRAITS INDIVIDUELS DANS UNE POPULATION NATURELLE DE TAMIAS RAYÉS

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DEDICATION

À Pascal

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RÉSUMÉ

Le microbiote intestinal varie selon les espèces hôtes, les individus et au cours du temps. Selon des études récentes, les communautés de microorganismes intestinaux peuvent avoir un impact important sur diverses fonctions de l'hôte, telles que le développement, l'immunité, la physiologie et le comportement. Dans la nature, les variations du microbiote intestinal entre les individus conduisent à des différences d'aptitude. De plus, des études récentes sur des souris et des rats de laboratoire ont révélé des relations entre les comportements liés à l'anxiété et la diversité et la composition du microbiote intestinal. Cependant, les études dans des contextes naturels sont rares, de sorte que les facteurs influençant la variation du microbiote dans les populations naturelles demeurent flous. L'objectif général de ce mémoire est d'étudier la variation individuelle du microbiote intestinal dans des conditions naturelles. Plus précisément, j'examine les dynamiques temporelles des microbes intestinaux et j'explore les liens entre le microbiote et les caractéristiques de l'hôte, avec un accent particulier sur le comportement. Nous avons suivi une population naturelle de tamias rayés (*Tamias striatus*) dans le sud du Québec en 2021 et 2022, et recueilli des échantillons fécaux pour estimer la composition bactérienne. Nous avons utilisé une approche de modèle mixte, des analyses de répétabilité et une partition de la variance des communautés microbiennes pour examiner les dynamiques du microbiote intestinal en utilisant plusieurs mesures de diversité. Nous avons montré que l'âge des tamias était un prédicteur significatif de la diversité-α du microbiote, et que la composition bactérienne intestinale variait en fonction du comportement et du site de capture. De plus, nous avons constaté que la diversité du microbiote était stable et hautement individualisée au sein d'une saison d'activité, mais que ces différences entre individus ne persistaient pas d'une année à l'autre. Nos résultats soutiennent la relation entre le microbiote intestinal et le comportement de l'hôte chez les mammifères et renforcent l'évidence croissante que l'individualité de la diversité et de la composition bactérienne dépend du temps.

Mots-clés : microbiome intestinal, écologie comportementale, variation individuelle, stabilité, répétabilité

SUMMARY

The gut microbiota varies across host species, individuals and time. Recent studies on animal microbiota have shown that gut microorganism communities can strongly impact diverse host functions, such as development, immunity, physiology and behaviour. Gut microbiota variations among individuals are hypothesized to lead to differences in fitness and ultimately, survival. Furthermore, recent studies on laboratory mice and rats have shown relationships between anxiety-related behaviours and gut microbiota composition and diversity. However, evidence in natural contexts is still scarce, such that factors driving microbiota variation in wild populations remain unclear. The general objective of this master's thesis is to investigate individual variation of the gut microbiota in nature. Specifically, I examine the temporal dynamics of gut microbes and explore the links between microbiota and host characteristics, with a particular focus on behaviour. We monitored a population of wild eastern chipmunks (*Tamias striatus*) in 2021 and 2022. in southern Québec, and collected faecal samples estimate bacterial composition. We used a mixed-model approach, repeatability analyses and variance partitioning of microbial communities to examine the dynamics of the gut microbiota using multiple diversity measures. We showed that age was a significant predictor of microbiota α-diversity, and that gut bacterial composition varied with behaviour and capture site. Additionally, we found that microbiota diversity was stable and highly individualized within a trapping season, but that among-individual differences mostly did not persist from one year to the next. Our findings support the relationship between gut microbiota and host behaviour in wild mammals and add to the growing evidence that individuality of bacterial diversity and composition is time dependant.

Key words: gut microbiome, behavioural ecology, individual variation, stability, repeatability

CHAPTER 1
GENERAL INTRODUCTION

1.1 Host-microbiome evolution

The associations between microbes and vertebrate hosts have a deep evolutionary history, shaped by millions of years of co-evolution, resulting in highly specialized and interdependent relationships (Ley et al., 2008; McFall-Ngai et al., 2013). Dynamic assemblages of bacteria, viruses, archaea, fungi, and other microbial eukaryotes, collectively known as the microbiota, inhabit various niches within and on the bodies of their hosts (Berg et al., 2020; Macke et al., 2017). Among these, bacteria have been the most extensively studied due to their abundance, diversity, and established relevance to human health (Sonnenburg et al., 2004). I will refer to the bacterial microbiota in my thesis.

Recent technological advances and the development of sequencing tools for studying microbial communities have opened new and unexplored research frontiers for ecologists. This burgeoning field has attracted significant attention, with research focusing on the evolutionary processes linking animals and microbes (Amato, 2013; Coyte et al., 2015; Suzuki, 2017; Kohl, 2019). The intimate relationship between hosts and their microbiota has led some authors to argue that crucial information about animal biology is missed if microbial communities are ignored (Zilber-Rosenberg & Rosenberg, 2008; Gilbert et al., 2012; Miller et al., 2018). For instance, Rosenberg et al. (2007) introduced the concept of the hologenome, proposing that hosts and their associated microorganisms should be studied as a single biological entity. Similarly, Miller et al. (2018) recommended applying metacommunity theory to address scale-related issues in microbiome science and better understand microbiome variation. These frameworks suggest that understanding the consequences of host-microbiome interactions could provide new insights into the proximate mechanisms driving ecological processes given the significant effects that microbes have at multiple ecological levels.

1.2 What is so special about the gut microbiota?

In vertebrates, the densest and most diverse microbial communities are in the gastrointestinal tract. (Hooper & Gordon, 2001; Donaldson et al., 2016; Williams & al., 2020; Cusick et al., 2021). Whether permanently established or just passing through, gut microbes compete for nutrients and space, producing metabolites that directly alter the physiochemical properties of their environment (Zhang et al., 2016). Hence, the gut microbiota has a critical impact on diverse hosts functions, affecting health.

One of the most well-known effects of mammalian gut microbes is their prominent role in host nutrition. These microbes enable access to nutrients that would otherwise be limited or unavailable. For instance, gut bacteria break down and convert plant fibers into short-chain fatty acids. These fatty acids are then assimilated by the host, regulating numerous metabolic pathways and serving as a significant source of energy (Flint & Bayer, 2008; LeBlanc et al., 2017; Amato, 2016). Bacterial symbionts also induce changes in gut motility and permeability (Muller et al., 2014; reviewed in Osadchiy et al., 2019), regulate energy extraction and storage (Bäckhed et al., 2004) and facilitate nutrient absorption (Neish, 2009).

Additionally, the gut microbiota plays an important role in the regulation of immune functions. The intestinal epithelium acts as a protective barrier while still maintaining homeostatic tolerance for mutualist or commensal microorganisms (McFall-Ngai et al., 2013; Margolis et al., 2021). A healthy and diverse gut microbiome helps the host resist pathogenic colonization via competitive exclusion (Costello et al., 2012; Pickard et al., 2017). Gastrointestinal symbionts also help attenuate inflammation (Kelly et al., 2004) and the development and differentiation of CD4+ T cells, which are crucial for determining host health status (Wu & Wu, 2012).

Although the gut microbiota is particularly known for its impacts on host nutrition and immunity, it also affects many other physiological systems though neuroendocrine signaling. Bacterial metabolism by-products can modulate host neurotransmitter and hormone levels and their precursors (Johnson, 2020). For example, gut-derived metabolites act on enterochromaffin cells in the digestive tract lumen and modify the bioavailability of serotonin, thereby regulating many host functions such as intestinal motility and secretory reflexes (Ridaura & Belkaid, 2015; Yano et al., 2015; Williams et al., 2020). Clearly, the diverse functions fulfilled by gut microbes confer extended metabolic abilities for their host across multiple pathways. Consequently, from an ecological point of view, the gut microbiota plays an essential role in host survival and fitness (Suzuki, 2017; Moran et al., 2019; Cusick et al., 2021).

1.3 Factors influencing the gut microbiota

The mammalian gut microbiota is first acquired during the passage through the birth canal, through maternal milk and via close physical contact with parents (Zilber-Rosenberg & Rosenberg, 2008; Mallott & Amato, 2021). However, many factors influence the ensuing microbial dynamics. These factors are often separated into host intrinsic or biological host factors, and extrinsic or environmental factors, which depend on external sources of contamination. Considering the wide-ranging effects of gut microbiota on hosts, many researchers aim to identify the source of gut microbiota variation in vertebrates.

1.3.1 Intrinsic factors

The gut microbiota varies according to several internal host factors that cannot be voluntarily modified. Host genetics account for a significant proportion of the variability seen in microbiota composition among individuals (Turnbaugh et al., 2009; Benson et al., 2010; Goodrich et al., 2014). Genetic factors influence many aspects of the gut environment, such as mucus composition, production of antimicrobial peptides, and general gut barrier integrity, which ultimately determines if it possesses the necessary substrates and resources for microorganisms to subsist. For example, the *Turicibacter* genus is under genetic control in humans (Goodrich et al., 2016) and mice (Benson et al., 2010; Org et al., 2016), with its abundance depending on the expression of a gene associated with bile acid composition (Kemis et al., 2019).

Age significantly impacts gut microbiota diversity and composition across various animal taxa (Yatsunenko et al., 2012; Xu & Zhang, 2021; Sadoughi et al., 2022). These changes likely arise from physiological variations, dietary shifts, and behavioural changes. Mammals are typically born with few gut bacteria, gradually accumulating species through stages of bacterial succession, with initial colonists originating from maternal vaginal microbes and breast milk (Palmer et al., 2007; Koenig et al., 2011; Bäckhed et al., 2015). For instance, juveniles host distinct and less diverse microbial communities compared to adults in wild baboons (*Papio cynocephalus*; Ren et al., 2016), in Brandt's voles (*Lasiopodomys brandtii*; Xu & Zhang, 2021) and in humans (Yatsunenko et al., 2012). Conversely, in red squirrels (*Tamiasciurus hudsonicus*; Petrullo et al., 2022), gut bacterial diversity does not change from youth to adulthood, while it decreases with age in chimpanzees (*Pan troglodytes schweinfurthii*; Degnan et al., 2012). In summary, the effects of aging on the gut microbiome appear to be species-specific and even study-specific in several host taxa.

Research has shown that gut microbiota can vary according to the host's sex, although findings are sometimes inconsistent. Physiological and behavioural differences are thought to drive variations in gut microbiota diversity and composition between females and males (Kim et al., 2020; Valeri & Endres, 2021). For example, sex-specific patterns have been shown to emerge due to hormone-microbe interactions, such as higher estrogen levels in females stimulating IgA secretion, which subsequently affects gut microbiota (Markle et al., 2013; Org et al., 2016; Sylvia & Demas, 2018; Pace & Watnick, 2021). In addition, several aspects of a species' ecology can lead to differential microbial inoculation between sexes in the wild (Bolnick et al., 2014; Corl et al., 2020). Life history processes, parental energy investment, social behaviour, diet and pathogen susceptibility are all ecological factors that can significantly influence gut microbial dynamics and often vary between males and females (Amato, 2013).

1.3.2 Extrinsic factors

Despite the clear influence of host genetics, age and sex in shaping gut microbial communities, several extrinsic factors also play a significant role in determining which microorganisms are encountered throughout the host's lifetime. A separate body of research has emphasized that the external environment's contribution to shaping gut microbiota often outweighs the influence of endogenous host factors in wild populations (Amato, 2013; Ren et al., 2017; Schmidt et al., 2019). Among these external factors, diet and surrounding environment are likely the most important drivers of vertebrate gut microbiota (Ley et al., 2006; Wu et al., 2011; Hird et al., 2015; Zhou et al., 2016; McKenzie et al., 2017; Leeming et al., 2019; Liddicoat et al., 2020; Teyssier et al., 2020).

Diet is a principal determinant of gut microbiota, as it provides a direct route to the gastrointestinal tract and creates selective pressures that shape microbial community structure (Amato, 2013; Ecklu-Mensah et al., 2022). Ingested food supplies essential nutrients that gut microbes utilize and metabolize, thereby influencing community dynamics (Leeming et al., 2019). For instance, in humans, gut microbiomes can rapidly adjust to dietary changes, with noticeable shifts in microbial composition and micronutrient intake occurring when individuals switch between animal-based and plant-based diets (David et al., 2014).

Direct exposure to microbes through soil, air, and other environmental elements also plays a critical role in shaping gut microbiota (Zhou et al., 2016; Mallott & Amato, 2021; Raulo et al., 2024). The structure and composition of the host's habitat determine the probability of colonization by filtering the microorganisms encountered in the environment, providing opportunities for microorganisms to establish and persist in the host (Ley et al., 2008; Amato, 2013; Moran & Sloan, 2015; Schmidt et al., 2019). For example, habitat type and large-scale landscape characteristics correlate with gut microbiota composition in howler monkeys (*Alouatta pigra* and *Alouatta palliata*; Amato et al., 2016) and in deer mice (*Peromyscus maniculatus,* Jameson et al., 2020), respectively. At finer spatial scales, interactions with different environmental substrates can generate distinct gut microbial communities, as observed in *Drosophila* species (Chandler et al., 2011), laboratory mice (Ottoman et al., 2019; Zhou et al., 2016), and in wild wood mice (*Apodemus sylvaticus*; Raulo et al., 2024).

While diet and habitat selection are partially under host control, other external factors, such as seasonal shifts in temperature, humidity, and pathogen prevalence, also modify the environmental pool of microbes, and consequently, the host's gut microbiota (Baniel et al., 2021; Kartzinel et al., 2019; Liu et al., 2023). These conditions can alter habitat structure and the availability, abundance, and diversity of resources, thus changing the microbial landscape that hosts are exposed to (Bletz et al., 2016; Shapira, 2016)

1.4 Gut microbiota and behaviour

The relationship between gut microbiota and host physiological homeostasis has attracted considerable attention in recent years. The substantial role of gut microbiota in maintaining brain function has been demonstrated in numerous studies over the past two decades (reviewed in Mayer et al., 2015). Gastrointestinal microbes can induce neurochemical changes in the brain by affecting the central nervous system, thereby altering host cognition and behaviour. These changes can have important ecological implications in wild animal populations (Archie & Tung, 2015; Suzuki, 2017; Davidson et al., 2018).

1.4.1 Bidirectional communication between the gut microbiota and the brain

Multiple signalling mechanisms underly the bidirectional communication between the gut microbiota and the brain. The gut-brain axis links the gastrointestinal tract to the central nervous system through neural, immune, and endocrine pathways (Figure 1.1; Mayer et al., 2015; Carabotti et al., 2015). Key elements of the gut-brain axis include the vagus nerve, which provides a direct neural link between the gut and the brain; the enteric nervous system, which processes and transmits information about gut conditions to the brain and regulates gastrointestinal activity; and the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine system involved in stress response (Margolis et al., 2021; Kasarello et al., 2023).

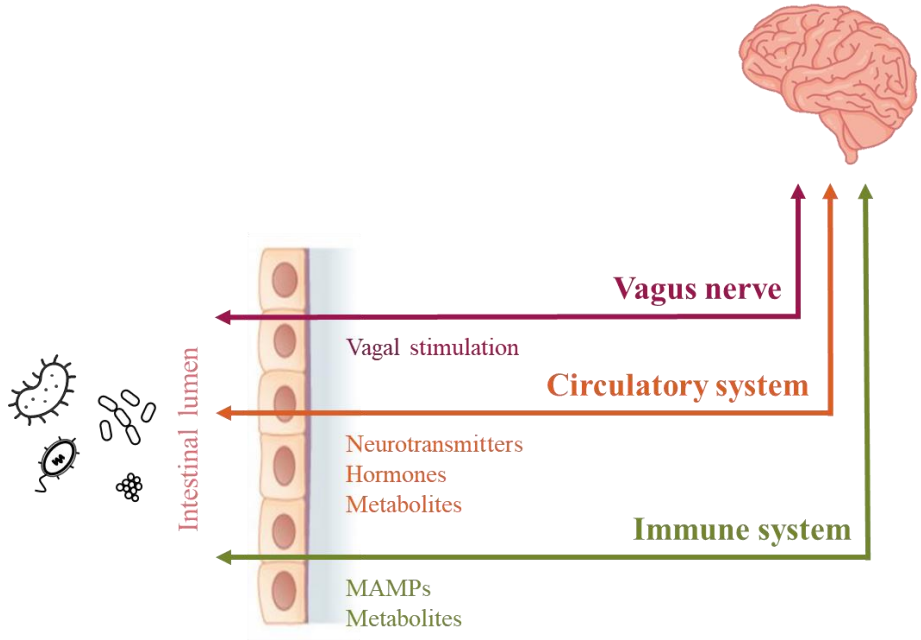

Figure 1.1 Main pathways linking the gut microbiota and the central nervous system. Adapted from Sampson & Mazmanian (2015).

Imbalances of the gut's bacterial composition alters several physiological systems, thereby affecting brain function and behaviour (Cryan & Dinan, 2012; Foster & Neufeld, 2013). Research indicates that the gut microbiota has an impact on HPA axis activity, affecting stress responses and inducing anxiety-like behaviours (Sudo et al., 2014; Martin et al., 2018; Williams et al., 2020; Cusick et al., 2021). When a stressor is detected, multiple signals travel through the different constituents of the HPA axis. First, the hypothalamus releases corticotropin-releasing hormone, which stimulates the pituitary gland to secrete adrenocorticotropic hormone (Carabotti et al., 2015). This hormone then prompts the adrenal glands to produce cortisol (in humans, fish, and most mammals) or corticosterone (in rodents, birds, amphibians, and reptiles), which are the primary stress hormones (Sudo, 2014; Carabotti et al., 2015; Williams et al., 2020).

Different methods have been used to assess the link between anxiety-related behaviours and the gut microbiota, either with the use of germ-free animals (Heijtz et al., 2011; Neufeld et al., 2011), antibiotic treatments (Jameson et al., 2020; Bercik et al., 2011), or faecal transplantation (Collins et al., 2013). Germ-free animals lack any bacterial contamination and offer the possibility to directly investigate the effects of inoculation on behaviour (Cryan & O'Mahony, 2011; Cryan & Dinan, 2012). Neufeld and colleagues (2011) have found that mice devoid of a microbiota show decreased anxiety-like behaviours compared with specific pathogen-free mice. Many other experiments using germ-free animals show similar results (Heijtz et al., 2011; Clarke et al., 2012). Additionally, Bercik et al. (2011) and Jameson et al. (2020) found that antibiotic treatment increases exploration in BALB/c mice and wild deer mice (*Peromyscus maniculatus*), respectively. Anxiety level is frequently assessed using measurements related to exploratory behaviour (Foster & Neufeld, 2013; Sampson et al., 2015; Davidson et al., 2018). A propensity to explore rapidly a novel environment and to stay in the center of an open space is generally a sign of low anxiety state (Belzung, 1999; Gould et al., 2009; Foster & Neufeld, 2013; Kraeuter et al., 2019). All these results seem to indicate that gut microbiota play an important role in determining the expression of anxiety-related behaviours, and in turn, behaviour can modulate which microbes are encountered in the environment.

1.4.2 Host behaviour shapes gut microbiota

Studies on nonhuman animals have shown relationships between gut microbial communities and various behaviours, such as aggressiveness (Sylvia et al., 2017), social behaviour (Archie & Tung, 2015), exploratory behaviour and anxiety (Bercik et al., 2011; Bravo et al., 2011; Heijtz et al., 2011; Foster & Neufeld, 2013). Since a significant portion of the gut microbiota is derived from the environment and food resources (Moran & Sloan, 2015), behaviours associated with foraging, such as exploration speed, could affect which microbes can be encountered and picked-up from the environment. In wild populations,

exploration and anxiety-related behaviours are involved in individual differences in foraging strategies (Spiegel et al., 2017), diet (Herath et al., 2021; Gharnit et al., 2022), pathogen transmission (Boyer et al., 2010; Webber & Willis, 2020) and habitat use (Boon et al., 2008; Pearish et al., 2013), all of which can modulate microbial exposures.

In nature, food resources are often scattered and heterogeneously distributed, and animals differ in the way they acquire them (Bolnick et al., 2003; Dall et al., 2012). Various behavioural traits influence space use patterns and foraging activity (Toscano et al., 2016; Spiegel et al., 2017; Erixon et al., 2024, in review). For instance, Patrick et al. (2017) found that bolder wandering albatross (*Diomedea exulans*) tend to be on the exploration end of the exploration-exploitation trade-off, performing less area-restricted search and covering greater distance between food patches. Similarly, bolder golden-mantled ground squirrels (*Callospermophilus lateralis*; Aliperti et al., 2021) and bank voles (*Myodes glareolus*; Schirmer et al., 2019) maintain larger core areas than their shyer counterparts. Moreover, hormones, such as cortisol and corticosterone, can also regulate locomotor and foraging activity in response to changing environmental conditions (Wingfield, 2003; Hau & Goymann, 2015; Spiegel et al., 2017).

Theory also suggests that individuals who remain thorough in their exploration would be slower to acquire resources (Toscano et al., 2016) and have smaller home ranges (Spiegel et al., 2017). Likewise, it is expected that different foraging tactics emerging from contrasting behavioural phenotypes can affect types of prey consumed (Toscano et al., 2016), since individuals favouring exploration over exploitation have a higher chance of encountering a larger breadth of prey. Gharnit et al. (2022) found that diet varies with foraging tactics in a population of eastern chipmunks (*Tamias striatus*). Individuals at both ends of the exploration spectrum tend to specialize in their prey choices, while moderate explorers had a more generalist diet. In edible dormice (*Glis glis*), the probability of finding unpredictable food sources increased with exploration (Wirowska et al., 2024). Therefor, behaviour can affect the likelihood of encountering novel microbes through direct contact with environmental objects and the ingestion of a different spectrum of prey.

Differences in foraging behaviour can also affect the probability of encountering diseases or parasites. Boyer et al. (2010) showed that Siberian chipmunks (*Tamias sibiricus*) with higher explorative tendencies are more likely to have a higher parasite load. Behavioural phenotypes can, therefore, indirectly affect animal health by influencing the probability of infection, thereby impacting gut microbiota.

1.4.3 Individual variance and animal personality

It has long been documented that individuals differ in multiple ways in a population (Dall et al., 2012). However, much of the work in psychology and biology in the 20th century put aside individual differences

and assumed that variation between subjects was due to uncontrolled factors (Clark & Ehlinger, 1987). Similarly, behavioural ecologists treated intraspecific variation as a maladaptation of individuals around an optimal strategy (Magurran, 1993). Over the last decades, the role of behavioural differences in evolutionary biology has become clearer. We now recognize the importance of taking an individual-based approach because behavioural differences between individuals are linked to differences in fitness (Smith & Blumstein, 2008; Moiron et al., 2020), and that natural selection occurs at this ecological level (Bolnick et al., 2011; Westneat et al., 2014; Toscano et al., 2016).

The concept of animal personality refers to among-individual differences in behaviour that are maintained over time (Réale et al., 2007; Carter et al., 2013). Multiple behavioural continua have been investigated in animal personality research, including activity level, boldness and exploration (Réale et al., 2007). Exploratory behaviour informs on how individuals react to a novel environment and acquire new information about their surroundings. According to the pace-of-life syndrome hypothesis, which stems from life history theory, those behaviours covary with a set of physiological traits, such as HPA axis and parasympathetic system reactivity, which also modulate gut microbiota communities (Amato, 2013; Macke et al., 2017). We could thus think that the metabolic mechanisms used to maintain individual differences in gut microbiota in a population are the same as those maintaining behavioural differences, and that microbiota parameters covary with behaviour (Foster & Neufeld, 2013; Davidson et al., 2020; Jameson et al., 2020a, Jameson et al., 2020b).

Furthermore, animals can vary in their response at different instances for a labile phenotypic trait. This within-individual variation can have substantial impacts on the biology that occurs at higher hierarchical levels (Westneat et al., 2014). For instance, the individual degree of endocrine flexibility can influence the expression of behaviours that are required to survive or reproduce, which can ultimately impact population dynamics (Taff & Vitousek, 2016). For example, in tree swallows (*Tachycineta bicolor*), females that can regulate glucocorticoid levels in response to different contexts fledge more offspring (i.e., have higher reproductive success) because they can better balance the demands of parental care with their stress response (Vitousek et al., 2018).

Ultimately, investigating both within- and among-individual physiological and behavioural differences, in relationship with microbiota variation, is crucial for understanding the subtle but influential mechanisms that drive ecological processes and shape evolutionary outcomes (Figure 1.2).

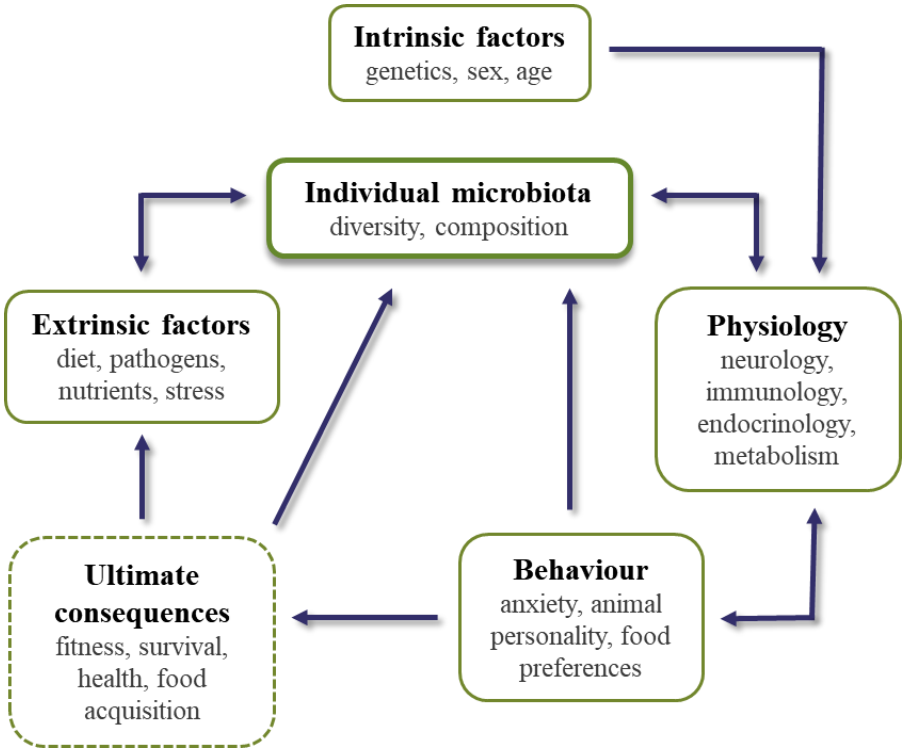

Figure 1.2 Causes and consequences of individual variation in the diversity and composition of the gut microbiota in relation to extrinsic (environmental effects) and intrinsic factors, and the subsequent impact on host physiology and behavioural traits. Adapted from Davidson et al. (2018).

1.5 Temporal dynamics of the gut microbiota

Temporal stability in gut microbial communities is considered crucial for maintaining host health, as it supports consistent physiological functions (Coyte et al., 2015). While changes in gut microbiota diversity, composition, and stability are anticipated across different life stages, these communities tend to remain relatively stable during adulthood (Lozupone et al., 2012; Faith et al., 2013; Valeri & Endres, 2021). However, few studies on the within-individual dynamics of gut microbes have been done on wild animals despite the important role of the microbiota on host fitness. On shorter time scales, wild gut microbiota can fluctuate in response to seasonal variations, leading to temporal shifts in gut microbiota parameters (Liukkonen et al., 2023; Marsh et al., 2022; Maurice et al., 2015; Orkin et al., 2019; Risely et al., 2022). Acting as a physiological transducer of environmental cues, the gut microbiota's rapid bacterial generation time enables faster host acclimation to stimuli (Alberdi et al., 2016; Hird, 2017; Cusick et al., 2021). Thus, evaluating the mechanisms that maintain gut microbiota balance, along with its responses to disturbances from changing environmental conditions, could provide critical insights into the adaptive potential of animal

species and the role of gut microbes in expanding hosts' ecological ranges (Moran et al., 2019; Greene et al., 2020; Moeller & Sanders, 2020).

Despite the temporal dynamics shown by the gut microbiota, growing evidence suggests that differences in the microbiota between individuals within a population persist across time. Indeed, numerous studies have highlighted a large effect of host identity on the microbiome, often referred to as individuality, over various time scales, ranging from two months to two decades (Degnan et al., 2012; Ren et al., 2017; Risely et al., 2022; Raulo et al., 2024; Somers et al., 2023). Individual signatures in gut microbiota likely arise from several factors that influence microbial selection. Physiological filtering, where host physiological traits selectively filter microbes, plays a crucial role in maintaining individuality (Mallott & Amato, 2021). For example, the composition of gut mucins and antimicrobial peptides, which are controlled by genetic expression, can create niches for specific microbial taxa (Liévin-Le Moal & Servin, 2006). The frequency and intensity of social contacts within host species can also modulate the transmission rate and convergence of the gut microbiota. Individuals with limited social interactions may have more distinct microbiota profiles compared to those with frequent social contacts, as seen in primate social networks (Amato et al., 2017; Tung et al., 2015). Ultimately, high levels of horizontal transmission, where microbes are acquired from the environment or other individuals, can reduce individuality by introducing various microbial taxa in a host's microbiota and by increasing community similarity among interacting individuals. Nevertheless, the extent to which individuality is maintained in natural populations, especially in changing environments, remains unclear.

1.6 Studying gut microbiota in wild populations

Laboratory-based studies have provided valuable insights into the relationship between gut microbes and host physiology, yet these controlled environments and the narrow spectrum of captive species offer only a limited perspective. Captive conditions fail to account for the complexity of natural environments, where varying ecological pressures and fluctuating resources shape the gut microbiota in ways that are not replicated in the lab (Cusick et al., 2021). It has also been shown that captive animals' gut microbiota differs from their wild counterparts (Hird et al., 2017; McKenzie et al., 2017; Davidson et al., 2020), In natural settings, hosts are exposed to a broader array of environmental microbes, dietary resources, social interactions, and other biotic factors, all of which contribute to the dynamic nature of the gut microbiome. Thus, investigating gut microbiota in wild, non-model organisms is crucial for capturing the full spectrum of ecological influences that drive within- and among-individual variation in these microbial communities.

While studying wild animal populations presents challenges, such as the difficulty of inferring causality from observational data, it allows for the exploration of microbiota-behaviour relationships in ecologically relevant contexts. Microbiota-behaviour studies often involve germ-free and specific pathogen-free animals, standardized diets, and controlled environmental exposures—conditions that do not reflect the dynamic and heterogeneous environments faced by wild animals, which can lead to misleading conclusions (Cusick et al., 2021; Davidson et al., 2020). Furthermore, the gut microbiota can influence host behaviour in ways directly tied to survival and reproductive success, making it imperative to study these dynamics outside of controlled laboratory conditions.

However, gut microbiota sample acquisition in wild populations presents significant challenges. Studying individual variance requires a longitudinal approach with replicate sampling of individual study subjects. Capturing wild animals while maintaining non-invasive methods involves additional ethical considerations. Moreover, limiting contamination and properly preserving fecal samples can be difficult. Despite these hurdles, longitudinal microbiome research is essential for understanding the ecological and evolutionary factors that shape gut microbiota, their adaptive significance, and their roles in host fitness.

Thus, our understanding of the stability and individuality of gut microbial communities in wild populations, as well as its relationship with host behaviour, remains incomplete. Studying host-microbiota relationships in natural habitats can reveal how individual microbiomes are maintained or altered over time, at multiple ecological scales (Hird, 2017; Davidson et al., 2020; Cusick et al., 2021).

1.7 Objectives

For my project, I am interested in within-individual (stability) and among-individual (individuality) variation of components of gut microbiota and its link to host traits in a natural population. I especially focus on the contribution of behaviour in shaping gut microbiota. My thesis is therefor divided into three main objectives:

1. Assess the relationships between the gut microbiota and host characteristics in a natural population. I present how gut microbiota diversity and composition are structured within a population according to host age, sex and multiple behavioural traits.
2. Evaluate the stability of the gut microbiota. I analyse how stable gut microbiota diversity and composition are within individuals throughout one active season.
3. Quantify individuality of the gut microbiota over multiple timescales.

I present the relative contribution of host identity in shaping the gut microbiota, and how it is maintained throughout one active season and across two years.

1.8 Study system

The eastern chipmunk (*Tamias striatus*) is a diurnal rodent in the Sciuridae family found throughout most of eastern North America (Elliot, 1978; Snyder, 1982). Eastern chipmunks are active above ground during the day and hoard seeds in their individual burrow. These burrows serve multiple purposes, including resting, sleeping, overwintering, and raising offspring. Chipmunks are solitary but often interact with conspecifics through agonistic interactions while defending their burrow or competing for resources (Couchoux et al., 2021). They have a relatively circular and symmetric home range centered around their burrow system (Yerger, 1953). During winter, eastern chipmunks undergo several torpor bouts, relying on hoarded resources between periods of activity (Munro et al., 2008).

The ecology of the chipmunk population in the Mounts Sutton in Southern Quebec is closely tied to a two-year-long masting cycle of the red maple (*Acer rubrum*) in spring and of the American beech (*Fagus grandifolia*) in fall (Bergeron et al., 2011*a*; Dubuc-Messier et al., 2012). In the spring of mast years, population density is at its lowest and the red maple seeds is their primary food source (Tissier et al., 2020; Gharnit et al., 2022). Chipmunks reproduce in the summer and juveniles emerge in early fall, coinciding with the production of beech seeds, they disperse and attempt to find a suitable burrow. This short period is critical for all chipmunks since they must amass enough food to sustain themselves throughout the winter. They also stock additional reserves and avoid dependence on the perishable and ephemeral resources the next spring, such as plant bulbs (e.g., trout lily, *Erythronium americanum* and Carolina springbeauty, *Claytonia caroliniana*), mushrooms, and invertebrates (Tissier et al., 2020). An additional breeding period occurs in early spring following a mast, with juveniles from this cohort emerging in May, leading to a peak in population density. Chipmunks must now wait a whole year for the next red maple mast and summer reproduction. Consequently, the breadth of their diet varies among individuals, between years, and in response to resource abundance (Gharnit et al., 2022). In the context of this study, year 2021 was a mast year, and 2022 was a non-mast year.

The pulsed resource dynamics also generate interannual variation in behaviour. Gharnit et al. (2022) showed an association between individual exploration profiles and multiple dimensions of the spatial niche, such as diet specialisation. These factors are closely linked to measurable aspects of the gut microbiota, offering insights into how it might vary across the population.

The long-term monitoring of the southern Quebec population has been ongoing since 2005. Each year, from May to September, morphological, behavioural, and genetic data are collected. The three capture sites (45° 05′ N; 72° 25′ W; Figure 1.3) are situated within 10 km of each other, and there are no significant differences in genetic structuring between these three subpopulations (Chambers & Garant, 2010). At each site we set a trapping grid with Longworth traps alternately placed at 40 m intervals along linear transects. Chipmunks are baited with peanut butter, live-trapped, and identified using metal ear tags (*National Band and Tag Co*., Newport, KY, U.S.A.) or with a pit tag (*Trovan Ltd.*, Alberta, Canada).

This study system is exceptionally well-suited for investigating individual variation in the microbiota and its relationship with behaviour. First, it is possible to collect samples with minimal invasiveness. Feces can be directly taken in the trap after manipulating the chipmunk. Secondly, the systematic tagging of individuals allows for repeated sampling over time, allowing for the monitoring of changes in microbiota within the same individuals across different seasons or years. Lastly, the same behavioural tests, such as the open-field test, have been used in numerous studies on this population over the years, revealing consistent individual differences (i.e., personality) in activity and exploration patterns (Montiglio et al., 2012; Santostefano et al., 2021; Gharnit et al., 2022). The ongoing use of these behavioural assessments improves our ability to link specific microbiota profiles to distinct behavioural traits.

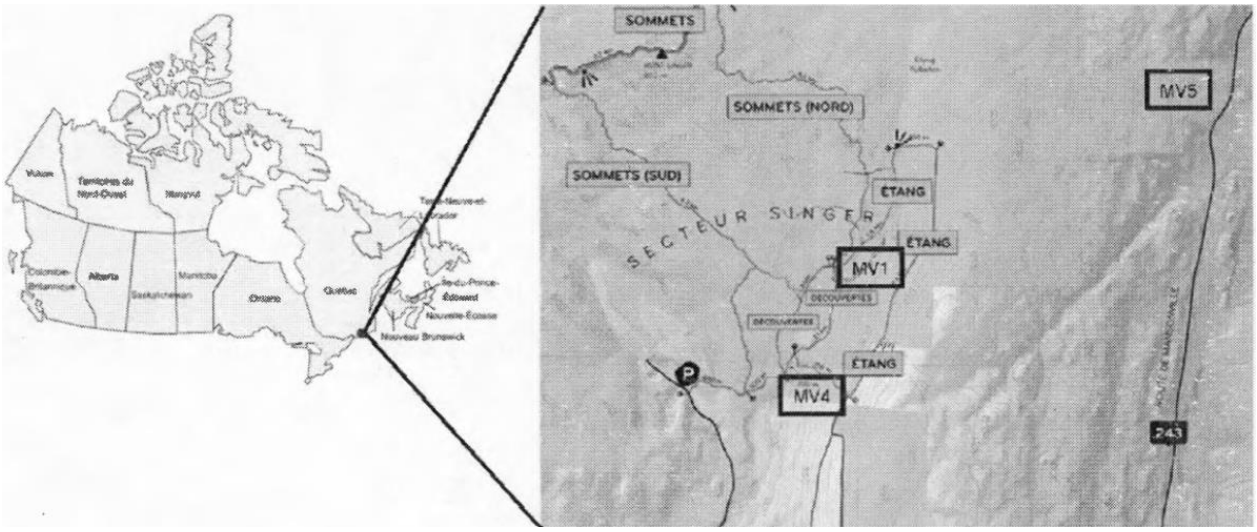

Figure 1.3 Geographic location of the three sampling sites, site 1 (MVl: 45°06. 785'/72°26.078'), site 2 (MV4: 45°06.207'/72°26.207') and site 3 (MV5: 45°07.852'/72°23.735') in the Mounts Sutton, Quebec, Canada.

CHAPTER 2
GUT MICROBIOTA'S TEMPORAL DYNAMICS AND RELATIONSHIPS WITH INDIVIDUAL TRAITS IN A WILD EASTERN CHIPMUNK POPULATION

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2.1 Abstract

The composition and diversity of the gut microbiota are known to impact host biological processes, and variation among individuals is hypothesized to lead to differences in fitness. However, studies of the gut microbiota in natural contexts are still scarce, and the factors driving individual microbiota variation in wild populations remain unclear. In this study, we sampled the gut microbiota of wild eastern chipmunks (Tamias striatus) over two years to investigate host-related factors influencing microbiota α-diversity and composition. We assessed the relative contribution of individual identity, and evaluated the stability of gut microbial communities. We amplified 16S rRNA gene sequences to quantify the bacterial community composition of 436 faecal samples. Our results revealed that juvenile chipmunks exhibited lower bacterial α-diversity compared to adults. Furthermore, the composition of the gut microbiota varied depending on chipmunk behavioural traits, where high exploration speed and centrality were linked to different microbial communities than those associated with trappability and trap diversity within an active season. Additionally, microbiota diversity was stable and highly individualized within a trapping season, though among-individual differences did not persist from one year to the next. Our findings support the relationship between gut microbiota and behaviour in wild mammals and add to the growing evidence that the individual signature of bacterial diversity and composition is time dependant.

2.2 Introduction

The gut microbiome is of paramount importance to animals (Berg et al., 2020; Ley et al., 2008). Microbial communities of the gut impact multiple physiological processes including immune function, nutrient acquisition and modulating behaviour, and thus play a fundamental role in host survival and fitness (Amato, 2013; Suzuki, 2017). While research in laboratory settings provides valuable insights into host-microbiota relationships under controlled conditions, these environments lack the complexity and variability of natural habitats. Laboratory studies often involve germ-free and specific pathogen-free animals, standardized diets and controlled environmental exposures, which are not representative of the dynamic and heterogeneous conditions faced by wild animals (Cusick et al., 2021; Davidson et al., 2020). In contrast, studying gut microbiota dynamics in natural populations captures the effects of diverse ecological factors, offering a more complete understanding of their roles in host ecology and evolution (Amato, 2016; Hird et al., 2017; Koskella et al., 2017).

Within a host species, individuals vary substantially in the composition of their microbiotas. Previous research has shown that sex, age and genetics drive gut microbial variation (Couch & Epps, 2022; Goodrich et al., 2014; Turpin et al., 2016). For instance, sex-specific patterns often arise due to hormone-microbe interactions (Markle et al., 2013; Sylvia & Demas, 2018). Age-related differences are also prominent, with juveniles typically hosting a distinct and less diverse microbial community compared to adults, as observed in species like wild baboons (Papio cynocephalus; Ren et al., 2016) and Brandt's voles (Lasiopodomys brandtii; Xu & Zhang, 2021). These age-related differences likely stem from physiological changes, dietary shifts, and behavioural changes that occur as individuals mature. Although gut microbial communities can exhibit strong temporal dynamics, growing evidence suggests individual differences in the microbiota within a population persist over time. Numerous studies have highlighted a large effect of host identity on the microbiome, often called "individuality", over various timescales, ranging from two months to two decades (Degnan et al., 2012; Ren et al., 2017; Risely et al., 2022; Raulo et al., 2024; Somers et al., 2023).

Individual signatures in gut microbiota likely arise from several factors that influence microbial selection. Physiological filtering, where host physiological traits selectively filter microbes, plays a crucial role in maintaining individuality (Mallott & Amato, 2021). For example, the composition of gut mucins and antimicrobial peptides, which are controlled by genetic expression, can create niches for specific microbial taxa (Liévin-Le Moal & Servin, 2006). The frequency and intensity of social contacts within host species can also modulate the transmission rate and convergence of the gut microbiota. Individuals with limited social interactions may have more distinct microbiota profiles compared to those with frequent social contacts, as seen in primate social networks (Amato et al., 2017; Tung et al., 2015). Ultimately, high levels of horizontal transmission, where microbes are acquired from the environment or other individuals, can

reduce individuality by introducing a diverse array of microbial taxa and increasing community similarity among interacting individuals. Nevertheless, the extent to which individuality is maintained in natural populations, especially in changing environments, remains unclear.

Some recent work supports the idea that, in wild populations, the contribution of environmental factors in shaping the gut microbiota commonly outweighs the influence of endogenous host factors (Amato, 2013; Ren et al., 2017; Schmidt et al., 2019). Diet and direct exposure to environmental microbes through soil and air are key drivers of vertebrates' gut microbiota (Hird et al., 2015; Leeming et al., 2019; Teyssier et al., 2020; Wu et al., 2011). While diet and habitat selection are partially under host control, other exogenous factors modify the gut microbiota, such as seasonal changes in temperature, humidity, and pathogen prevalence (Baniel et al., 2021; Kartzinel et al., 2019; Liu et al., 2023). These seasonal shifts can generate changes in habitat structure, and in resource availability and diversity, thereby altering the environmental pool of microbes. Consequently, in natural populations, hosts' gut microbiota is expected to vary over time and across space in response to seasonal fluctuations (Liukkonen et al., 2023; Marsh et al., 2022; Maurice et al., 2015; Orkin et al., 2019; Risely et al., 2022). However, our understanding of the temporal stability of gut microbe communities (within-individual variation of the microbiota) in wild host species remains limited, primarily due to the difficulty of acquiring repeated samples of the same individuals over extended periods (Orkin et al., 2019). Longitudinal studies are essential to understand how the gut microbiota is shaped by fluctuations in host state and in the environment.

The relationship between the gut microbiota and behaviour has attracted attention in the recent years. Gastrointestinal microbes can induce neurochemical changes in the brain by affecting the central nervous system, and consequently alter host cognition and behaviour (Davidson et al., 2020). For example, studies on nonhuman animals have shown relationships between gut microbial communities and aggressiveness (Sylvia et al., 2016), social behaviour (Archie & Tung, 2015), exploratory behaviour and anxiety (Bercik et al., 2011; Bravo et al., 2011; Diaz Heijtz et al., 2011; Foster & Neufeld, 2013). In wild populations, exploration and anxiety-related behaviours are involved in individual differences in foraging strategies (Spiegel et al., 2017), diet (Gharnit et al., 2022; Herath et al., 2021), pathogen transmission (Boyer et al., 2010; Webber & Willis, 2020) and habitat use (Boon et al., 2008; Pearish et al., 2013), which can alter and modulate microbial exposures. Still, the ecological relevance of the microbiome-gut-brain axis remains largely unexplored.

Descriptive data on wild gut microbiomes are starting to accumulate, but longitudinal studies incorporating behavioural measures remain scarce. Here, we studied eastern chipmunks (Tamias striatus) from a wild population in southern Québec, Canada, to (1) investigate how the gut microbiota diversity varied with individual hosts characteristics (sex, age and behaviour) and environmental factors within a single year of

activity, (2) examine the stability of the microbiota over that same period, and (3) quantify the relative contribution of host identity (individuality) in shaping gut microbe communities over one year and across years.

Eastern chipmunks in southern Québec have been studied extensively for nearly two decades and represent an ideal system to expand on previous research on host-microbiome relationships. They experience major shifts in food resource availability due to the heavily seasonal environment and the among-year fluctuation in seed production by dominant deciduous trees, resulting in contrasting population densities from one year to another (Bergeron et al., 2011; Tissier et al., 2020). Specifically, the ecology of chipmunks largely depends on a two-year-long masting cycle of the red maple (Acer rubrum) and American beech (Fagus grandifolia; Bergeron et al., 2011a; Tissier et al. 2020). Although they consume a variety of plants, mushrooms and invertebrates (Tissier et al., 2020), the breadth of their diet varies among individuals and with resource abundance and availability (Gharnit et al., 2022). During the spring of mast years, population density is at its lowest and the red maple is their main food source (Tissier et al., 2020; Gharnit et al., 2022), while during the fall, chipmunks predominantly harvest and store American beech tree seeds in their burrow for the winter. Population density usually peaks in the spring following a mast year. In this study, 2021 was a mast year, while 2022 was a non-mast year.

The pulsed resource dynamics also generate interannual variation in behaviour. Gharnit et al. (2022) showed that individual differences in exploration profiles are associated with multiple dimensions of spatial niche use, such as dietary specialization. Furthermore, individuals in this population show consistent differences in several behaviours across time or through situations (Gharnit et al., 2022; Montiglio et al., 2012; Santostefano et al., 2021).

Thus, we expected that individual traits, such as sex, age and behaviour, would play a significant role in shaping gut microbiota diversity and composition due to their interactions with host physiology and ecology. We also hypothesized that while individuality will be evident in the microbiota within a single year, the persistence across years would be weakened given the strong effects of masting events on resource availability. Finally, we expected the individual microbiota to remain stable across time points within a year. By addressing these objectives, our study seeks to clarify the relative contributions of host identity, intrinsic and extrinsic factors in shaping the gut microbiota of wild mammal populations.

2.3 Methods

2.3.1 Study system and sites

We live-trapped eastern chipmunks (Tamias striatus) at three sites of 6.76 ha (site 1 and 2) and 3.24 ha (site 3), located in a deciduous forest of Mounts Sutton, southern Québec, Canada (45° 05′ N; 72° 25′ W). All three sites are less than 10 km apart and the three subpopulations are genetically identical (Chambers & Garant, 2010). At each site we established a trapping grid with Longworth traps alternately placed at 40 m intervals along linear transects.

Eastern chipmunks are active above ground during the day and hoard seeds in their individual burrows, which they use to rest, sleep, overwinter, raise their offspring, and that they defend against conspecifics. Although solitary, chipmunks often engage in agonistic interactions while defending their burrows or competing for resources (Couchoux et al., 2021). They have a relatively circular and symmetric home range centred around their burrow system (Yerger, 1953). At our study sites, home range sizes are stable with an average diameter of 37 m for females and vary between a diameter of 47 m in non-mast years to 90 m in mast years for males (Montiglio, 2009). Additionally, the immediate social environment can influence the phenotype of an individual for life history, behaviour, and morphological traits (Santostefano et al. 2021), which may subsequently affect the spatial distribution of gut microbes across sampling sites.

2.3.2 Sample collection

We conducted daily trapping from May to mid-September in 2021 and from May to mid-August in 2022. Traps were baited using peanut butter and checked every two hours, from 8:00 a.m. until 4:00 p.m. Upon first capture, we marked individuals with metal ear tags with a unique code (National Band and Tag Co., Newport, KY, U.S.A.) and a pit tag (Trovan Ltd., Alberta, Canada). At each capture, we recorded the identity, the sex, and determined the age class (juvenile or adult; Bergeron et al. 2011b). We also collected stool samples from the traps using sterilized tweezers after handling and identifying each chipmunk. We put faecal pellets in RNAlater Stabilization Solution (Thermo Fisher Scientific). We kept the samples in a cooler for a maximum of six hours then stored them at -20 °C. Four blank extractions were made throughout the season to check for potential contamination during the collection process.

In 2021, we took one sample per individual in the spring (May—June) and a second one in the late summer (mid-July—mid-September) if the chipmunk was trapped anew (139 individuals, 180 samples). In 2022, to analyse variation among individuals in the ASV richness and composition of the microbiota, we gathered three to six samples per individual throughout the season (May—mid-August), with at least six days

between the collection of each sample (61 individuals, 256 samples). We collected 436 samples from 172 individuals. Twenty-eight individuals were trapped in both years.

2.3.3 Behavioural assays

We used an open-field test (OF) to record horizontal locomotion and thigmotaxis/centrality as indices of chipmunk exploration speed and anxiety, respectively (Gharnit et al., 2020; Montiglio et al., 2010, 2012; Santostefano et al., 2021; Réale et al., 2007). Individual repeatability for exploration speed in the OF in this population has previously been estimated to 0.32 (Montiglio et al., 2010), 0.26 (Santostefano et al., 2021) and 0.41 (Gharnit et al., 2020). Montiglio et al., (2012) also found moderate repeatability for centrality ($r = 0.20$).

The OF tests were performed once a year, at the OF area, in the centre of the trapping grid, from the last week of May until the first week of July. Upon capture, we identified the trapped chipmunk, then kept it in the trap to carry it to the OF arena, which consisted of a wooden box with gridlines at the bottom and a transparent plexiglass lid. Before going into the OF arena, we transferred the chipmunk into a small chamber connected to the OF where it stayed for 60s. We then videotaped its behaviour for 90 s. The OF was cleaned with 70% ethanol after every test. For each test, we analysed the videos and measured exploration as the number of lines crossed ("exploration speed") and anxiety as the proportion of time spent in the centre of the arena ("centrality"; Montiglio et al., 2012).

We used the number of times an individual was trapped during the active season ("trappability") as a proxy for above-ground activity throughout the active season, and the number of different traps used ("trap diversity") as a proxy for space use (Paquette et al., 2020). In chipmunks, trap diversity and trappability are related to increased infestation by ticks (Ixodes ricinus; Boyer et al. 2010), and bot flies (Cuterebra spp.; Paquette et al. 2020).

2.3.4 DNA extraction and sequencing

We used amplicon sequencing to quantify gut bacterial communities. DNA was extracted from the samples using Norgen Stool DNA Isolation Kits (Norgen Biotek Corp) following the manufacturer's instructions. The V4-V5 hypervariable regions of the 16S rRNA gene were amplified using the 515 F/926R universal bacterial primers (Walters et al., 2015) and sequenced on an Illumina MiSeq. PCR conditions were an initial denaturation at 98˚C for 30 s, 30 cycles of denaturation at 98˚C for 15 s, of primer annealing at 50˚C of 30 s, and of extension at 72˚C of 30 s, with a final extension of 10 min at 72˚C. Samples from the two years were sequenced on two separate runs. The library preparation, the sequencing and demultiplexing of sequence reads were conducted at the UQAM CERMO-FC Genomics Platform.

2.3.5 Data processing/Bioinformatics

All sequence reads were processed using the DADA2 pipeline (version 1.28.0; Callahan et al., 2016) in R (version 4.3.0; R Core Team, 2023). After visually assessing the quality profiles of the sequences, we retained nucleotides at positions 19–175 bp for the forward DNA sequences and positions 20–260 bp for the reverse sequences. We identified amplicon sequence variants (ASV) with the DADA2 function "dada" in pseudo-pooling mode and removed probable chimeras with the function "removeBimeraDenovo" using consensus pooling mode, resulting in a total of 8 939 647 sequences derived from 28 654 ASVs. The total number of sequences varied among samples, ranging from 3597 to 63 794 sequences per sample. We used the SILVA database (version 138.1; Quast et al., 2013) and the "assignTaxonomy" function to determine the taxonomic identity of all ASVs. We evaluated the mock community composition in the positive controls and subsequently excluded these samples from the dataset. Furthermore, we eliminated all non-bacterial sequences, totaling 39 411 sequences. We rarefied the dataset to 3800 sequences per sample using the R package phyloseq (version 1.44.0; McMurdie & Holmes, 2013), as this threshold was sufficient to capture the vast majority of ASVs in samples (Fig. S1). All negative controls were excluded except for one containing 7613 sequences, which was removed due to a predominance of Rhizobium, a common soil bacterium. The final dataset contained 6389 ASVs across 436 samples.

2.3.6 Data analysis

A preliminary analysis of the correlations between host variables showed that trap diversity increased with trappability (corr = 0.64). However, we decided to keep both variables in our models since they provide complementary information about the individual spatial ecology, and therefore could affect the gut microbiota differently. We considered the age category as an inherent host characteristic since the assigned age was fixed within yearly sampling period. Continuous predictor variables were centred and scaled before analyses.

To estimate sample α-diversity, we computed two complementary diversity measures: observed ASV richness (the count of ASVs per sample, hereafter referred to as richness) and the Shannon diversity index, which accounts for both richness and taxa abundance ("diversity" function from the using the vegan package; version 2.6-4; Oksanen et al., 2022). For each model, we first ran the model with richness as the response variable then with the Shannon index. All statistical analyses were conducted in R (version 4.3.0; R Core Team, 2023).

2.3.6.1 Host traits and within-year stability

Using data from 2022 only, we tested for the relationship between host characteristics and microbiota ASV richness and Shannon index. We also tested for potential individual differences in the richness and α-diversity throughout the summer. For this analysis we used two separate random slope models (Schielzeth & Forstmeier, 2009) with each diversity measure as the response variable, and we included chipmunk sex (female or male as factor), age (juvenile or adult as factor), exploration speed, centrality, trap diversity, trappability, capture day (Julian day), and site (sites 1-3 as factors) as fixed effects. The random slopes consisted of the interaction between sampling day (n = 48) and chipmunk identity as a random effect. We calculated the R^2 values using the *MuMIn* package (version 1.47.5; Bartoń, 2009).

To investigate the individual-level dynamics of α-diversity measures over time, we extracted the correlation coefficient between intercept and slope from our random slope models. This correlation coefficient reveals whether there is a systematic linear relationship between the initial microbiota diversity level (intercept) and the rate of change in diversity (slope) across individuals. A null intercept-slope correlation indicates that initial diversity levels do not predict the rate of change, though individuals can still exhibit wide variations in their slopes. These variations may be independent of initial diversity levels. To validate the patterns underlying the correlation, we examined the plot and assessed the model fit. Next, keeping the same fixed-effect structure, we used likelihood ratio tests (LRT) to compare the goodness of fit of the random-slope models (i.e., individuals differ in both their levels at the intercept and in their slopes) with random-intercept models (i.e., individuals differ in their levels at the intercept but not in their slopes) and a null model (i.e., no differences between individuals in both levels at the intercept and slopes).

To analyze the composition of the gut microbiota, we Hellinger-transformed the community matrix, conducted partial redundancy analyses (RDA) and partitioned the variance between groups of variables, with significance assessed using a permutation test (Legendre & Legendre, 2012). Analyses were done using the *vegan* package. The first partial RDA had the same structure as the α-diversity models, except the response variable was the transformed community matrix. We extracted the two ASVs that had the highest and lowest scores on the first three axes. We then used the function *varpart* to partition the variation in community data between four sets of variables: inherent characteristics (sex and age), behaviour (exploration speed, centrality, trap diversity, trappability), site and Julian day.

To verify potential relationships between host behaviour profile and gut bacterial phylum relative abundances, we first did a visual inspection on bar plots, with chipmunk identities ordered according to each behavioural measure. Also, we extracted the post-rarefaction abundance of the two main phylum (*Bacteroidetes* and *Firmicutes*) in adults and modelled them separately in a GLMM with a Poisson

distribution using the same fixed effects as the previous models to inspect taxon stability and relationships with explanatory variables.

2.3.7 Individuality of the gut microbiota diversity and composition

To estimate individuality of the microbiota through one trapping season, we calculated the repeatability (Nakagawa & Schielzeth, 2013) of the previous α-diversity models using the rptR package (Stoffel et al., 2017). To assess individuality in the gut microbiota composition, we ran a partial of chipmunk identity as the sole explanatory variable, while controlling for all the other host variables.

We tested for differences in gut microbiota α-diversity between years and estimated cross-year individual consistency in α-diversity. Using lme4 package (version 1.1-33; Bates et al., 2015), we first ran separate mixed-effect models with the two α-diversity measures as the response variable, with year (2021 and 2022), Julian day, age, sex and site as fixed effects, and chipmunk identity (2021, n = 139; 2022, n = 61) as a random effect, to explore patterns between years.

Secondly, to quantify individuality over the years and to control for differences in the sampling and age structure between 2021 and 2022, we selected the first sample of each year for all adults captured in both years (n = 28). Since we could not calculate repeatability due to the lack of replicated individual samples of the microbiota in 2021, we calculated the Pearson correlation coefficient between the two α-diversity measures from 2021 to 2022 to assess cross-year individuality. To investigate individuality in the composition of the gut microbiota from one year to another, we conducted a redundancy analysis on the bacterial community matrix with chipmunk identity and year as fixed effects, while controlling for variance between sites. The proportion of variance explained by chipmunk identity, and the adjusted repeatability measure ("RsquareAdj" function in vegan) provide insights into the persistence of individuality in the composition of microbe communities. Finally, we ran a Poisson GLMM with Bacteroidetes and Firmicutes abundances as the response variable, added year as a fixed effect, and calculated the repeatability to infer inter-year taxa individuality.

2.4 Results

2.4.1 Alpha diversity analysis

2.4.1.1 Host characteristics and traits

Adult and female chipmunks had significantly higher ASV richness and Shannon index values compared to juveniles and males, respectively (Table 2.1). The lower α-diversity in males was, however, driven by the samples of one juvenile chipmunk (see supplementary material, Figure S2.1).

Behaviours and sampling sites were not significant predictors of gut microbiota ASV richness or Shannon index (Table 2.1). However, when juveniles were removed from the dataset, site 2 showed a significantly lower richness than the two others (see Table S2.1). Richness increased with Julian day, but only when juveniles were included in the model. The fixed effects explained 17% (marginal R^2) of the variation in Shannon index, while the conditional R^2, which accounts for fixed and random effects, was estimated at 58%. The marginal and conditional R^2 of the richness model were 32% and 96%, respectively (Table 2.1).

Table 2.1 Effects of sex, age, site, Julian day and behaviour traits on the gut microbiota ASV richness and Shannon diversity index of our study chipmunk population in southern Québec, Canada, over an active season (2022). We used a mixed-effect model with random slopes. The marginal and the conditional R^2, for the richness model, were 0.32 and 0.96, respectively. For the Shannon model, the marginal and the conditional R^2, were 0.17 and 0.58, respectively. "Corr." corresponds to the correlation between the intercepts and the slopes.

	ASV richness			**Shannon**		
	Estimate (± SE)	*z-value*	*p-value*	Estimate (± SE)	*t-value*	*p-value*
Intercept	5.96 (± 0.04)	140.82	<0.001	4.85 (± 0.06)	80.24	<0.001
Site (Site 2)	-0.06 (± 0.06)	-1.04	0.30	-0.13 (± 0.10)	-1.32	0.19
Site (Site 3)	0.06 (± 0.06)	1.14	0.26	-0.04 (± 0.09)	-0.42	0.68
Julian day	**0.03 (± 0.07)**	**2.22**	**0.03**	0.03 (± 0.02)	1.30	0.20
Age (Juvenile)	**-0.19 (± 0.05)**	**-2.69**	**0.007**	**-0.36 (± 0.11)**	**-3.32**	**0.002**
Sex (Male)	**-0.12 (± 0.03)**	**-2.31**	**0.02**	**-0.19 (± 0.08)**	**-2.26**	**0.03**
Exploration	0.01 (± 0.03)	0.48	0.63	-0.04 (± 0.04)	-1.10	0.28
Centrality	0.01 (± 0.02)	0.53	0.60	-0.01 (± 0.04)	-0.37	0.71
Trap diversity	-0.02 (± 0.01)	-0.63	0.53	-0.03 (± 0.05)	-0.58	0.57
Trappability	-0.04 (± 0.02)	-1.18	0.24	-0.05 (± 0.05)	-1.04	0.30
	Variance	Corr.		Variance	Corr.	
ID (*Intercept*)	0.051			0.067		
Julian day	0.011	-0.71		0.003	-0.62	
Residuals				0.071		

n = 256, id = 61

2.4.2 Stability

At the population level, richness increased significantly with Julian day (Table 2.1). Including random slopes improved the fit of the model compared to the random intercept model (LRT $\chi^2 = 292.18$, $p < 0.001$, Fig 2.1A, Table S2.2). The correlation between intercept and slope in the richness model was strongly negative (-0.71, Table 2.1) meaning that individuals with low richness value at the average date showed greater increases in richness over time than individuals with high richness at this date (Fig. 2.1A).

The Shannon index did not change significantly with Julian day (Table 2.1). Adding random slopes to this model did not improve the fit of the data (LRT $\chi^2 = 1.89$, $p = 0.39$, Fig 2.1B, Table S2.2), indicating that slopes for Shannon index with time did not vary significantly among individuals. The addition of random intercepts improved the fit of a null model (LRT $\chi^2 = 46.74$, $p < 0.001$, Table S2.2). We, however, show the

random slope model (Fig 2.1B) to interpret the fixed effects and other random effects while controlling for pseudoreplication (Schielzeth & Forstmeier, 2009).

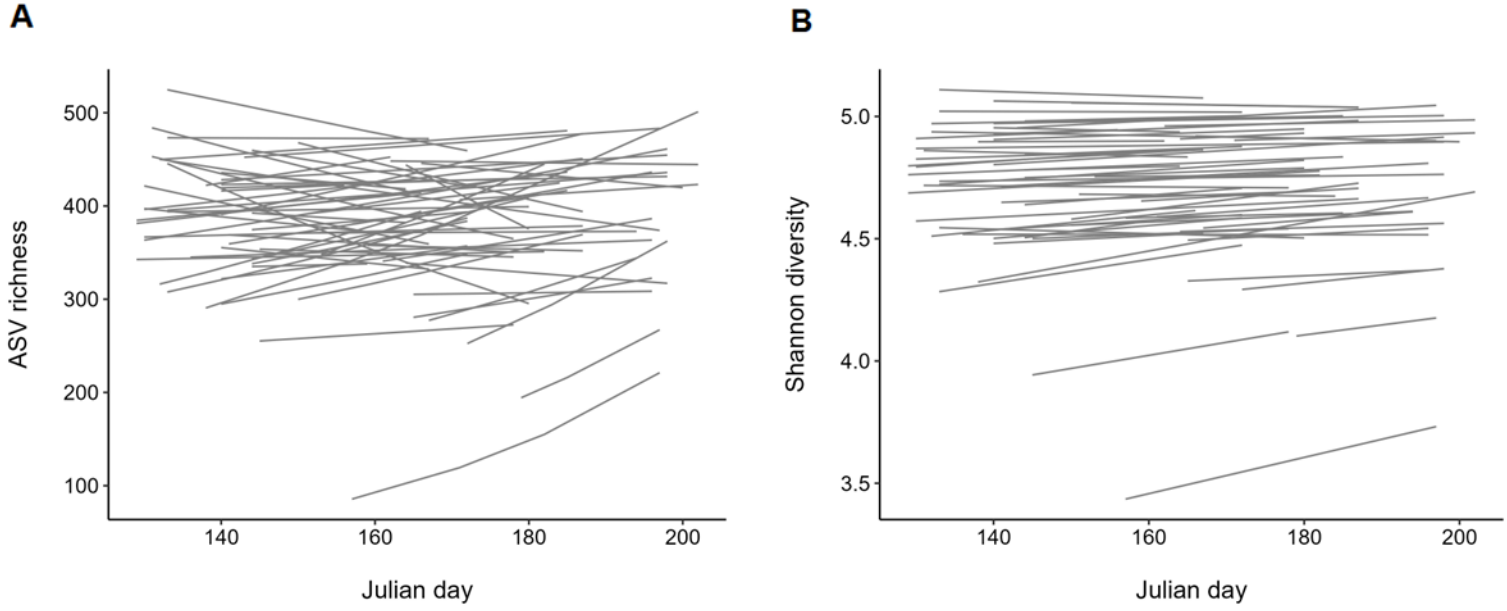

Figure 2.1 Predicted slopes for individual ASV richness and Shannon Index (n = 61) of the gut microbiota of eastern chipmunks from our study population in southern Québec, Canada, from May to July 2022. The predictions were adjusted for site 1, females, and at the mean of all host behavioural measures.

2.4.3 Individuality

In 2022, host identity explained most of the variance in both α-diversity models (Table 2.1). That year, we detected repeatable individual differences in ASV richness ($r = 0.46$, $p < 0.001$) and Shannon diversity index values ($r = 0.45$, $p < 0.001$).

Pearson correlations between the gut microbiota α-diversity measures across years were not significantly different from 0 (richness: Pearson $r = -0.24$, $p = 0.22$; Shannon: Pearson $r = -0.21$, $p = 0.29$).

2.5 Composition analysis

2.5.1 Taxonomic characterization and phylum level relative abundance

The eastern chipmunk gut microbiota was dominated by Bacteroidetes (73% of sequences), primarily from the Muribaculaceae and Prevotellaceae families, and Firmicutes (22% of sequences), mainly from the Lachnospiraceae family.

None of the host variables varied with Bacteroidetes and Firmicutes abundance. Additionally, we found no evidence for the stability of these taxa over the course of one year. However, we observed some individuality in Firmicutes abundance ($r = 0.20$) but not in Bacteroidetes abundance ($r = 0.05$). The inter-year models revealed that samples taken in 2022 contained a lower proportion of Bacteroidetes, with an estimated rate ratio of 0.98 ($SE = 0.005$, $z = -3.83$, $p < 0.001$, 95% CI: 0.97, 0.99). In contrast, chipmunks harboured on average more Firmicutes, with an estimated rate ratio of 1.03 ($SE = 0.009$, $z = 3.45$, $p < 0.001$, 95% CI: 1.01, 1.05; Fig. S2.2).

2.5.2 Variance partitioning

In the main redundancy analysis, we found that age, sex, behaviour, capture site and julian day explained 7.6% of the total variance in gut bacterial communities. The Venn diagram (Fig. 2.2) showed that most of the variance was not shared between variable types. The Julian day explained 0.9% of variance while sex and age explained 1.5%. Behaviours altogether explained 2.2% of variance. All four behaviours contributed somewhat equally to the variation of the microbiota, explaining between 0.57% and 0.73% of its composition (see Fig. S2.3). Differences in the microbiota between sites were responsible for 2.5% of variance.

Chipmunks from a same site hosted more similar gut bacterial communities than chipmunks from different sites (Fig. 2.3A). Site 1 and 2 were more similar than Site 3. Site 1 and 2 differed on the second axis of the ordination (RDA2), while Site 3 differed from the two other sites on the first axis (RDA1) (Fig. 2.3A).

2.5.3 Stability and individuality of the bacterial composition

In 2022, the microbiota composition showed little variation at the within-individual scale (Fig. 2.3B). Many ellipses in the ordination are small and narrow, illustrating a high stability of the bacterial community composition within individuals across samples. A few ellipses are apart and do not overlap with any other ellipse, suggesting that some individuals host distinct gut microbiota communities. The redundancy analysis with the chipmunk identity as the sole response variable indicated that 29.3% of variance in the gut

microbiota composition was attributed to differences between individuals, which denotes a degree of individuality in the composition of bacterial communities in this population.

Over a longer timescale (i.e., 2021 and 2022 combined), the composition of the individual microbiota weakly persisted from one year to another, with chipmunk identity accounting for 2.8% of variance in the bacterial communities ($R^2_{adj} = 0.028$, $p = 0.029$, Table S2.4). However, when analyzing the RDA ordination, we found that some chipmunks' microbiota tended to be very similar in both years (ex. -N052 and Q031), and that other individuals show different communities (ex. Q070 and Q090, Fig S2.4).

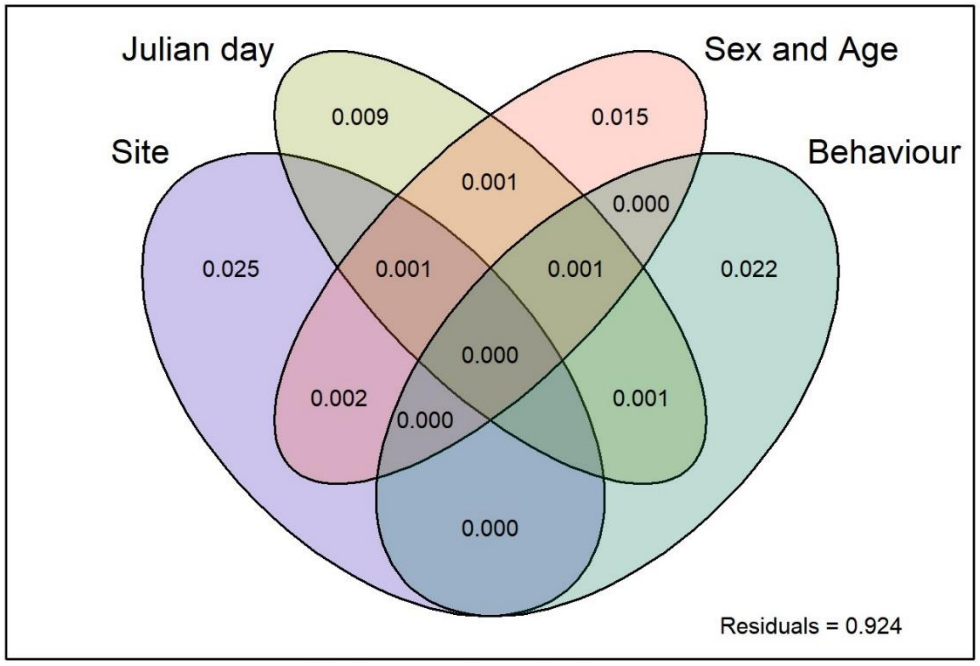

Figure 2.2 Proportion of variation in gut bacterial communities explained by site, Julian day, sex and age, and behaviour (including exploration speed, centrality, trappability and trap diversity) from May to July 2022 in eastern chipmunks from our study population in southern Québec, Canada.

2.5.4 Microbiota composition and behaviour

Given that the axes RDA2 and RDA3 explained similar amounts of variance (1.2% and 1.0%, respectively) and that *trappability* and *trap diversity* were barely represented by the second axis, we decided to analyse both ordinations jointly.

Fast explorers had high scores along the RDA1 axis (Fig. 2.3C), whereas slow thorough explorers were associated with low scores of this axis. Additionally, highly central chipmunks (low anxiety) had both high score along the RDA1 axis and a low score along the RDA2 axis. The genus *Treponema* (phylum *Spirochaetota*) and *Prevotella* (phylum *Bacteroidota)* are the two main taxa positively associated with the first axis. *Treponema* and bacteria from the *Muribaculaceae* family are also negatively associated with the second axis.

Microbial composition along RDA3 was associated trappability and trap diversity (Fig. 2.3D). The taxa negatively related to the third axis are the genus *Butyricimonas* and *Bacteroides*, both from the *Bacteroidota* phylum

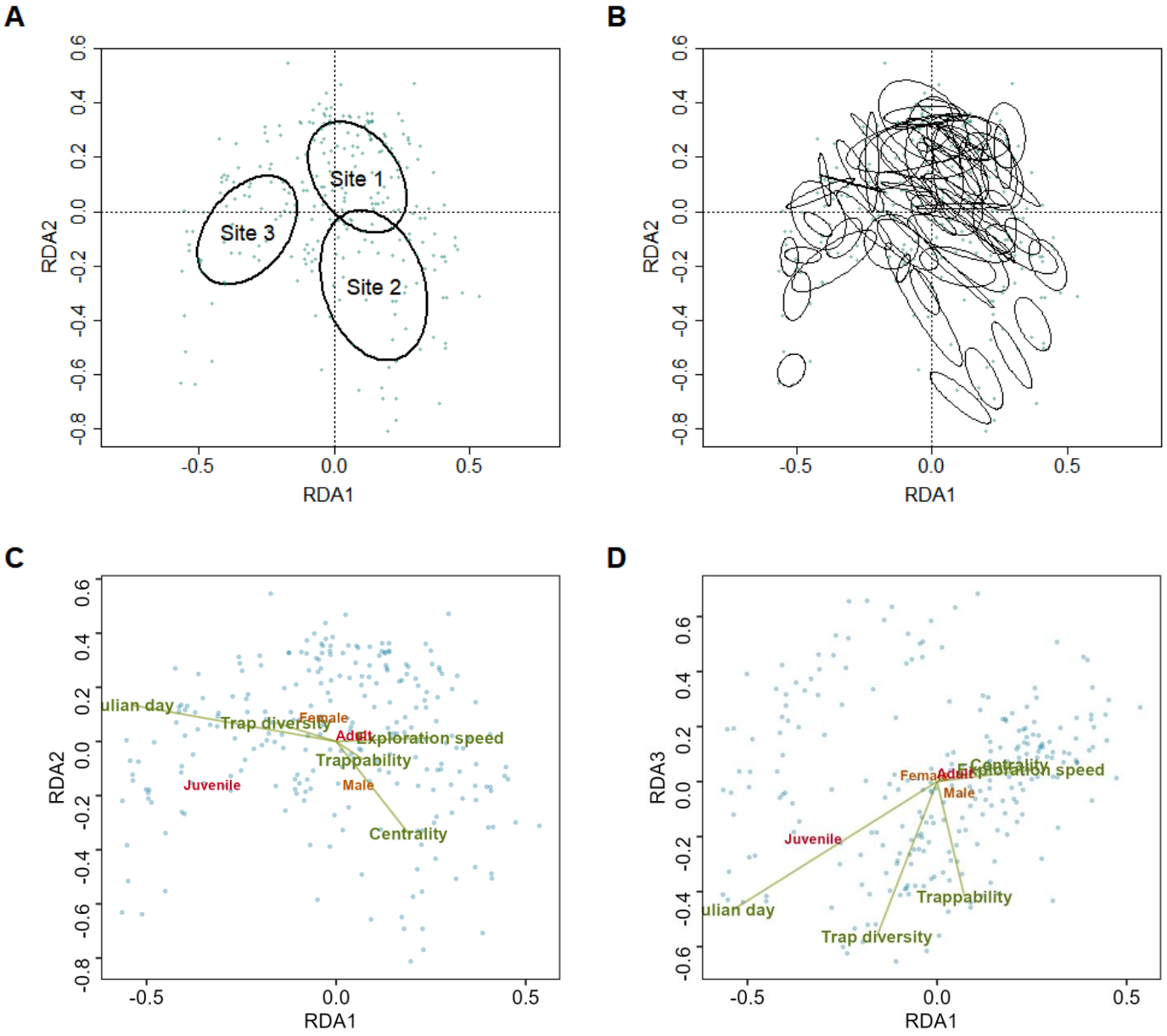

Figure 2.3 Redundancy analysis of gut microbiota samples of eastern chipmunks from our study population in southern Québec, Canada, through the active season (2022). Sites (A), individual ellipses (B), inherent characteristics and behaviours (C, D) are presented. The axes RDA1 and RDA2 are shown in C while D illustrates RDA1 and RDA3.

2.6 Discussion

In this study, we described the bacterial gut microbiota of wild eastern chipmunks over two years and analysed the relative contribution of host individual traits and environmental factors in shaping these communities. The taxonomic composition of the bacteria in the microbiota of wild Tamias striatus revealed a typical chipmunk gastrointestinal bacterial profile, dominated by Bacteroidetes and Firmicutes, consistent with previous findings (Grond et al., 2020; Zhou et al., 2022). We found evidence of an individual signature of the gut microbiota over the course of one year (2022) for all diversity measures. These differences among individuals did not persist across years for α-diversity, whereas the microbiota composition was slightly persistent from one year to another. Dynamics of ASV richness differed among individuals within a year, but the Shannon index remained stable. Additionally, age was a major factor that influenced both gut bacteria α-diversity measures. Although we did not find any relationships between α-diversity and behaviour, our results did reveal patterns with the composition of the microbiota. Specifically, we observed differing communities between two pairs of behaviours: exploration speed and centrality, and trappability and trap diversity. The largest portion of variance in microbiota composition was explained by sampling sites, followed by individual behaviour.

Previous studies have shown a large effect of host identity on gut microbiota (Degnan et al., 2012; Marsh et al., 2022; Ren et al., 2017; Turnbaugh & al., 2009). In our study, we found that both α-diversity and composition of the gut microbiota were moderately repeatable (i.e., 0.46 for richness, 0.45 for Shannon index and 0.29 for microbiota composition) within a year, indicating the presence of individualized microbial signatures in this population. However, when analysed over a longer timescale of two years, none of the differences between individuals in the microbiota α-diversity persisted, and individuality of the microbiota composition was reduced to 2.8%. Mallott (2022) recently highlighted that in most longitudinal studies of wild populations, individuality of the gut microbiota is time-dependent and decreases considerably over time. For instance, a two-decade study on wild meerkats (*Suricata suricatta*) by Risely *et al*. (2022) found that inter-year variation overrides the signal of individuality for samples collected more than two months apart. This pattern is expected in populations where horizontal transmission mechanisms, such as frequent social contact or strong environmental colonization, are predominant. At our study site, the two-year pulsed resource production cycles of dominant trees directly impacts both resources availability and population density, thus possibly leading to varying pools of environmental bacteria. This variation could overshadow the effects of individualized gut microbiota signatures in eastern chipmunks from one year to the next. Furthermore, during winter, chipmunks go through several torpor bouts, with important changes in their physiology, and feed on a restricted food reserve (Humphries et al., 2002). These conditions may promote the convergence of the microbiome across individuals between the end of the summer and

next spring, potentially reducing individuality of the gut microbiota in the population. Future research should investigate whether individuality persists when comparing ecologically analogous years in pulse systems as this could provide insight into the contribution of external factors in shaping the gut microbiota in the wild. Interestingly, some chipmunks exhibited very similar gut bacterial compositions over the years (e.g., N052 and Q031; Fig. S5) and showed a highly persistent α-diversity (Fig. S6). In contrast, another chipmunk (Q090; Fig. S5) had dissimilar communities between 2021 and 2022, with a significant increase in both α-diversity measures from one year to the next (Fig. S6). Conversely, another individual (R085; Fig. S5) also exhibited differing communities over the years but maintained a persistent α-diversity (Fig. S6). These observations suggest that the richness and Shannon index alone might be inadequate to measure gut microbiota individuality. Individuals with stable α-diversity could still experience partial turnover in their gut communities, which is not captured by traditional α-diversity metrics and could lead to misleading conclusions (Johnson & Burnet, 2016).

The consensus of a stable gut microbiota diversity through time has been challenged in the past decade (Ren et al., 2016; Risely et al., 2022; Sadoughi et al., 2022). Although the study population lives in a heavily seasonal environment, we found no evidence of an individual shift in Shannon diversity through time. However, chipmunks' microbiota showed different dynamics of richness over the summer: a few chipmunks hosted a high number of bacterial ASVs in early spring, followed by a steep reduction. The gut microbiota of most individuals maintained a stable ASV count around the population mean, while several individuals emerge in spring with a lower richness and gradually accumulate ASVs as time goes by. Given that ASV richness is accounted for in the calculation of the Shannon index, our findings suggest that while dominant taxa show stability over time, the dynamics of rare taxa differ among individuals of this population. Seasonal changes in chipmunk physiology such as previous reproductive activity status (either the preceding summer and/or early spring) or individual differences in winter torpidity cycles and emergence from hibernation might affect colonization of certain microbes. Indeed, Zhou et al. (2022) found that hibernation and arousal significantly altered both α-diversity and ß-diversity of the gut microbiota of Siberian chipmunks (*Eutamia sibiricus*), supporting the hypothesis of torpor-related shifts in the eastern chipmunk gut microbiome. Alternatively, the quantity and quality of burrow-hoarded food resource could also explain differences in richness in early spring, since the nutrient content of food resources (Carabotti et al., 2015) and fasting (Sonoyama et al., 2009) have been shown to alter the gut microbiota.

Unsurprisingly, we found that the α-diversity of chipmunks' gut microbiota was dependent on age: juveniles harboured on average a lower gut bacterial diversity than mature individuals. Multiple studies have found that the α-diversity tends to increase from early life to adulthood in many host taxa, including humans (Xu et al., 2019), wild baboons (*Papio cynocephalus*; Ren et al., 2016), barn swallows (*Hirundo rustica*;

Kreisinger et al., 2017), and Brandt's voles (*Lasiopodomys brandtii*; Xu & Zhang, 2021). This gain in diversity can be associated with a combination of external factors, including changes in diet and gradual exposure to novel environmental microbes; in our study system, trapped juveniles had few opportunities to expand the breadth of their gut microbiota. After emergence from their natal burrow, young disperse within the next two weeks and must find a burrow (Elliott, 1978), which tends to be in a low-quality area (Gaudreau-Rousseau et al., 2023). Fewer interactions with conspecifics, limited food resources, and the suboptimal microenvironment surrounding the available burrows could explain the reduced gut microbiota diversity observed in juvenile chipmunks.

Differing physiology or ecology between females and males in wild populations often lead to sex-dependent patterns in host gut microbiota (Stothart et al., 2019). Given that eastern chipmunks differ between sexes in their dispersal patterns (Dubuc-Messier et al., 2012), faecal cortisol concentration (Montiglio et al., 2012), behaviour and diet (Gharnit et al., 2022), we expected to find relationships with either diversity or composition of gut bacterial communities. We found differences in the gut microbiota α-diversity between female and male chipmunks, but that were entirely dependent on host age. This result may be due to the uneven sampling between male and female juveniles: our sample included only one juvenile male as opposed to eight juvenile females. Soon after emergence, young males disperse further from the natal burrow than females, which makes their long-term monitoring challenging. A more thorough sampling of juvenile chipmunks to avoid confounding effects, and integrating data on reproductive status and hormone fluctuation, could give important insight into how the gut microbiota varies among sexes in this population.

Relationships between behaviour and several parameters of the gut microbiota α-diversity have been established in humans (Johnson, 2020) and multiple animal species (Partrick et al., 2018; Florkowski & Yorzinski, 2023; Xia et al., 2024). In contrast, we found no evidence for a relationship between chipmunk behaviour phenotypes and the α-diversity of their gut microbiota in our study system. The contrast between the absence of effects of individual traits on microbial α-diversity and the strong repeatability of these two traits indicates that other unmeasured traits are probably related to such variation in microbial communities. Li et al. (2016) found that diet diversity in wild plateau pikas (*Ochotona curzoniae*) did not affect gut microbiota α-diversity, but was associated with b-diversity, suggesting no linear relationship between microbiota richness and diet breadth. In line with this finding, our redundancy analysis revealed that a considerable part of the among-individual variation in the composition of the microbiota was due to individual differences in behaviour. Although our experimental design prevented us from predicting causal effects between the microbiota and behaviour, it is interesting to note that while behavioural differences in this population are consistent through time (Gharnit et al., 2022; Montiglio et al., 2012; Santostefano et al.,

2021), the microbiota does not persist from one year to another. These results suggest that it is unlikely that gut microbes explain interindividual differences in behaviour.

Gut bacterial communities associated with exploration speed differed slightly from those associated with centrality. Multiple studies have found that animals with lower concentrations of faecal glucocorticoid metabolites (i.e., lowly anxious animals, fast explorers) were associated with an increase in the relative abundance of intestinal pathogens (Noguera et al., 2018; Petrosus et al., 2018; Petrullo et al., 2022). Given that host health and immune system activation can have important impacts on gut microbiota composition, fast explorers and highly central individuals could offer differing gastrointestinal conditions, and thus, host distinct communities. Our redundancy analysis also shows that gut microbiota composition is divided between two pairs of behaviours: exploration speed and centrality on RDA1, and trappability and trap diversity on RDA3. One possible explanation is that each pair reflect different characteristics of the host, each impacting a distinct dimension of the host-microbiota relationship. Exploration speed and centrality are both accepted measures of response to novelty and anxiety, respectively (Belzung, 1999; Kraeuter, 2019), which reflect the effects of host stress physiology on gut bacterial communities. Alternatively, trap diversity and trappability are behaviours that are mostly associated with the physical interactions between the host and its environment, independently selecting for other bacterial ASVs. These results highlight the multifaceted nature of the gut microbiota-host relationship, where different behavioural traits associated with physiological and environmental interactions contribute uniquely to shaping microbial communities in wild populations.

Although the microbiota was not significantly different among sites in our main α-diversity models, we found that adults had a lower richness in site 2. Our findings align with a prior study in voles, where gut microbiota α-diversity was correlated with landscape characteristics (Jameson, 2021). Interestingly, we observed a decrease in chipmunk activity on site 2 in the past four years. This site now corresponds to the site with the lowest chipmunk density. We hypothesize that beech bark disease might have affected more trees on site 2, reducing the quality of the habitat and chipmunk density. Furthermore, most of the variation of the gut microbiota composition in the population was explained by differences between sites, site 3 communities being distinct from sites 1 and 2. Although all sampling sites are located within 4 km from one another, site 3 is farther in distance and more isolated from the two other sites. This evident spatial structure suggests that microhabitats are a major driver of gut microbiome dynamics in the wild. This finding is consistent with what other studies have observed for wild red squirrels (*Tamiasciurus hudsonicus*; Ren et al., 2017) and house mice (*Mus musculus domesticus*; Goertz et al., 2019), where microbial composition varied at a small local scale. Additionally, site 3 has unique ecological factors that can contribute to changing

the available environmental pool of bacteria, such as higher humidity (Liu et al., 2023), increased abundance of conifers (Amato et al., 2016) and interspecific competitors such as red squirrels (He et al., 2018).

In summary, our study highlights the complex interplay between host traits, environmental factors, and gut microbiota composition and α-diversity in wild eastern chipmunks. While juvenile chipmunks exhibited lower bacterial α-diversity than adults, behaviour and sampling sites significantly influenced gut microbial communities. The individuality and stability of gut microbiota were evident within a single active season, but individuality diminished across the years, supporting that environmental factors have a major impact on gut microbial communities. Our findings underscore the need for further research into the temporal dynamics and ecological factors shaping the gut microbiota in wild populations.

2.7 Acknowledgements

We thank all field workers for their help with sample collection, as well as Pauline Van Leeuwen and Geneviève Bourret of UQAM CERMO-FC Genomics Core for their help with laboratory work and with analyses. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to DR, the Fonds de Recherche du Québec–Nature et technologies (FRQNT) team grant to PB for funding.

2.8 Supplementary materials

Table S2.1: Effects of sex, site, Julian day and behaviour traits on the gut microbiota ASV richness of the adults of a chipmunk population of Mounts Sutton in Québec, Canada, over an active season (2022).

	Estimate (± SE)	*z-value*	*p-value*
(Intercept)	5.977 (± 0.02)	241.346	0.000
Site (Site 2)	-0.104 (± 0.04)	-2.461	**0.014***
Site (Site 3)	0.053 (± 0.04)	1.267	0.205
Sex (Male)	-0.042 (± 0.04)	-1.187	0.235
Trap diversity	-0.022 (± 0.02)	-1.096	0.273
Trappability	0.002 (± 0.02)	0.073	0.942
Exploration	-0.020 (± 0.02)	-1.241	0.215
Julian day	0.012 (± 0.01)	1.055	0.291
Centrality	0.003 (± 0.02)	0.191	0.849
	Variance	Corr.	
ID (*Intercept*)	0.012 (± 0.11)		
Julian day	0.005 (± 0.07)	-0.31	

n = 224, id = 52

Table S2.2 α-diversity analysis – likelihood ratio tests

Response variable	Model type	χ^2	*p-value*
Richness	Null – RI	1930.90	<0.001*
	RI - RS	292.18	<0.001*
Shannon	Null – RI	48.62	<0.001*
	RI - RS	1.89	0.39

df = degrees of freedom, RI = random intercept model, RS = random slope model

Table S2.3 Effects of sex, age, site, Julian day and behaviour traits on the gut microbiota composition of a chipmunk population of Mounts Sutton in Québec, Canada, from May to July 2022.

Variable	F	*p-value*
Sex	3.51	<0.001*
Age	3.95	<0.001*
Site	4.47	<0.001*
Julian day	3.70	<0.001*
Exploration	2.45	<0.001*
Centrality	2.25	<0.001*
Trap diversity	2.62	<0.001*
Trappability	2.67	<0.001*

n = 256, id = 61

Table S2.4 Proportion of variance explained by host identity (ID) on the gut microbiota composition of a chipmunk population of Mounts Sutton in Québec, Canada, over two active seasons (2021-2022).

Variable	R^2_{adj}	*p-value*
ID	0.028	**0.029***

n = 52, id = 28

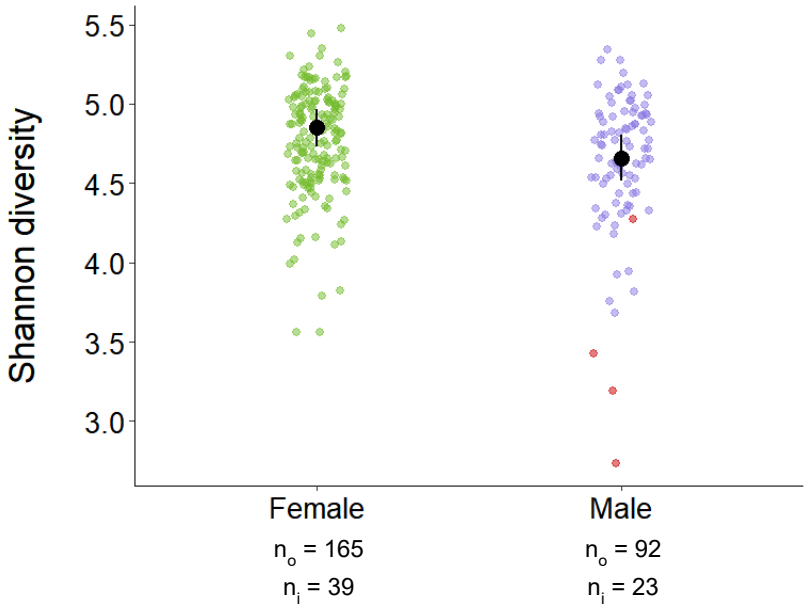

Figure S2.1 Mean Shannon diversity index for every sample according to sex. The male outlier individual's samples are highlighted in red.

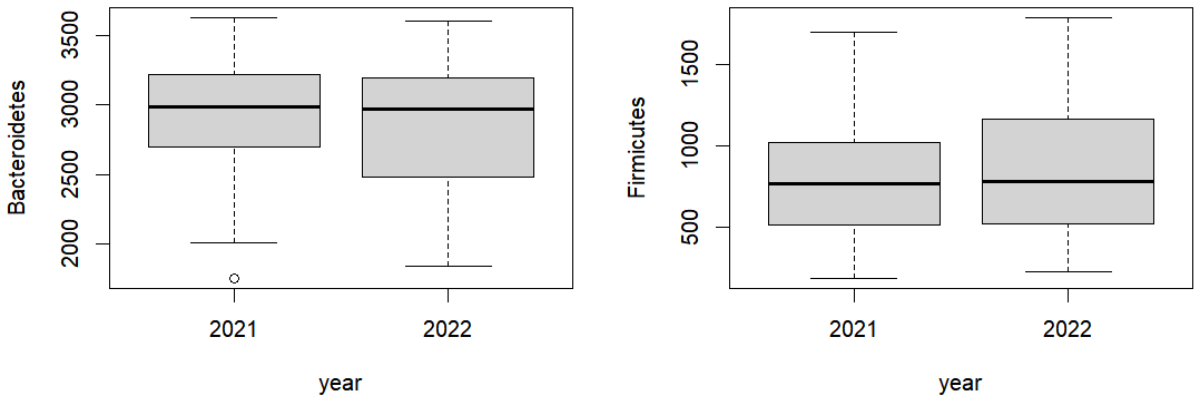

Figure S2.2 Abundance of *Bacteroidetes* and *Firmicutes* according to year. Mean Bacteroidetes abundance is higher in 2021 ($p < 0.001$) and mean Firmicutes abundance is higher in 2022 ($p < 0.001$).

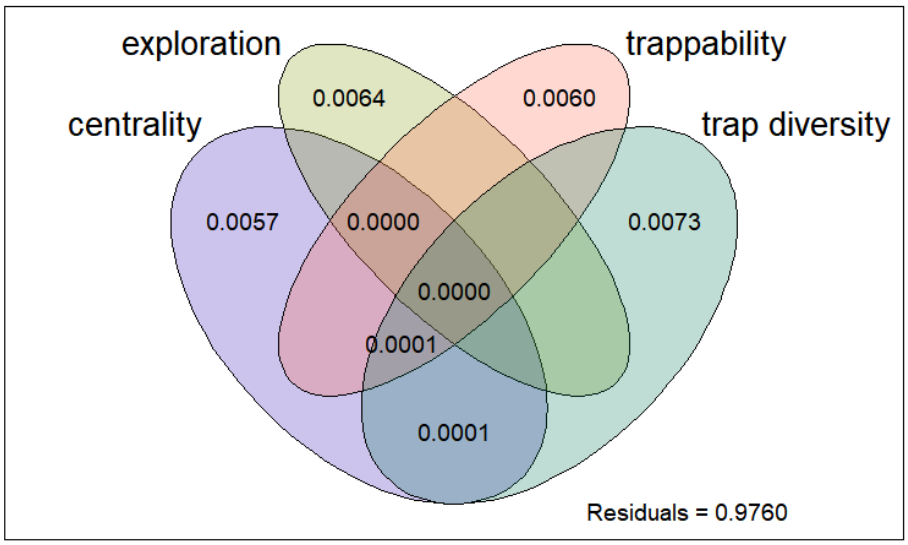

Figure S2.3 Proportion of variation in gut bacterial communities explained centrality, exploration speed, trappability and trap diversity) in a natural population of *T. striatus* of Mounts Sutton, Quebec, Canada, in 2022.

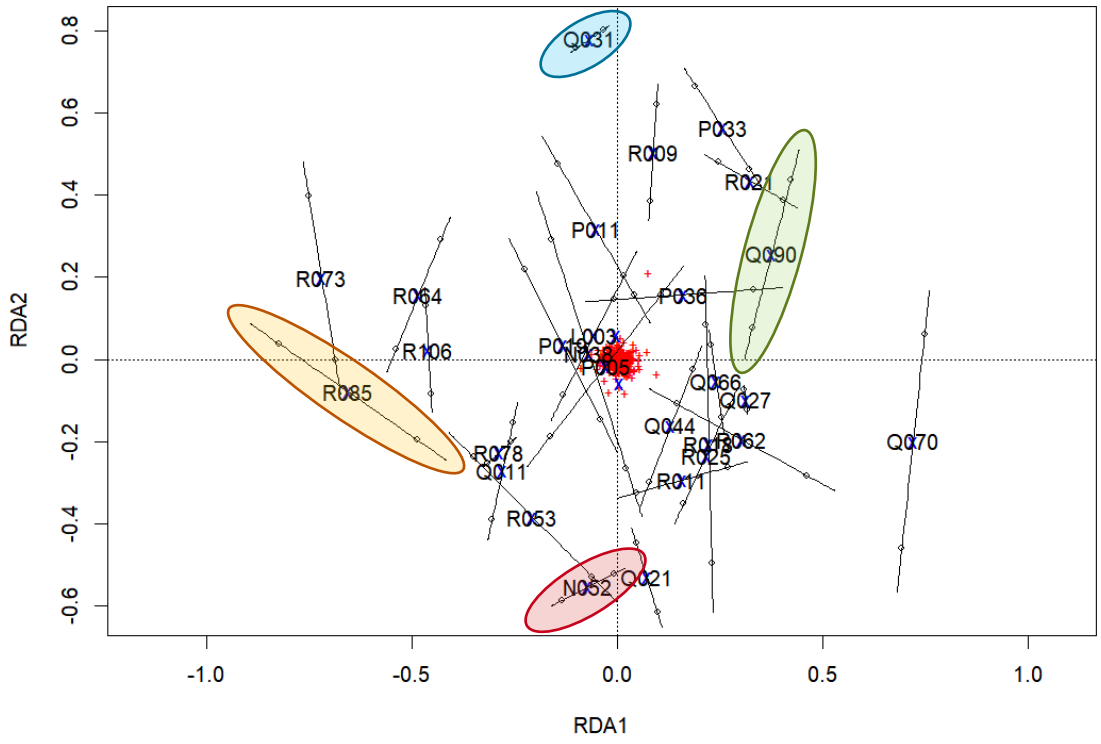

Figure S2.4 Inter-year individuality of the gut microbiota composition of eastern chipmunks (n = 28) of Mounts Sutton, Quebec, Canada. Two seemingly stable individuals, Q031 (blue) and N052 (red), and two other individuals that appeared to be variable in their composition between years, R085 (orange) and Q090 (green), were highlighted to do a comparative investigation between the composition, ASV richness, Shannon index and phylum relative abundance through time.

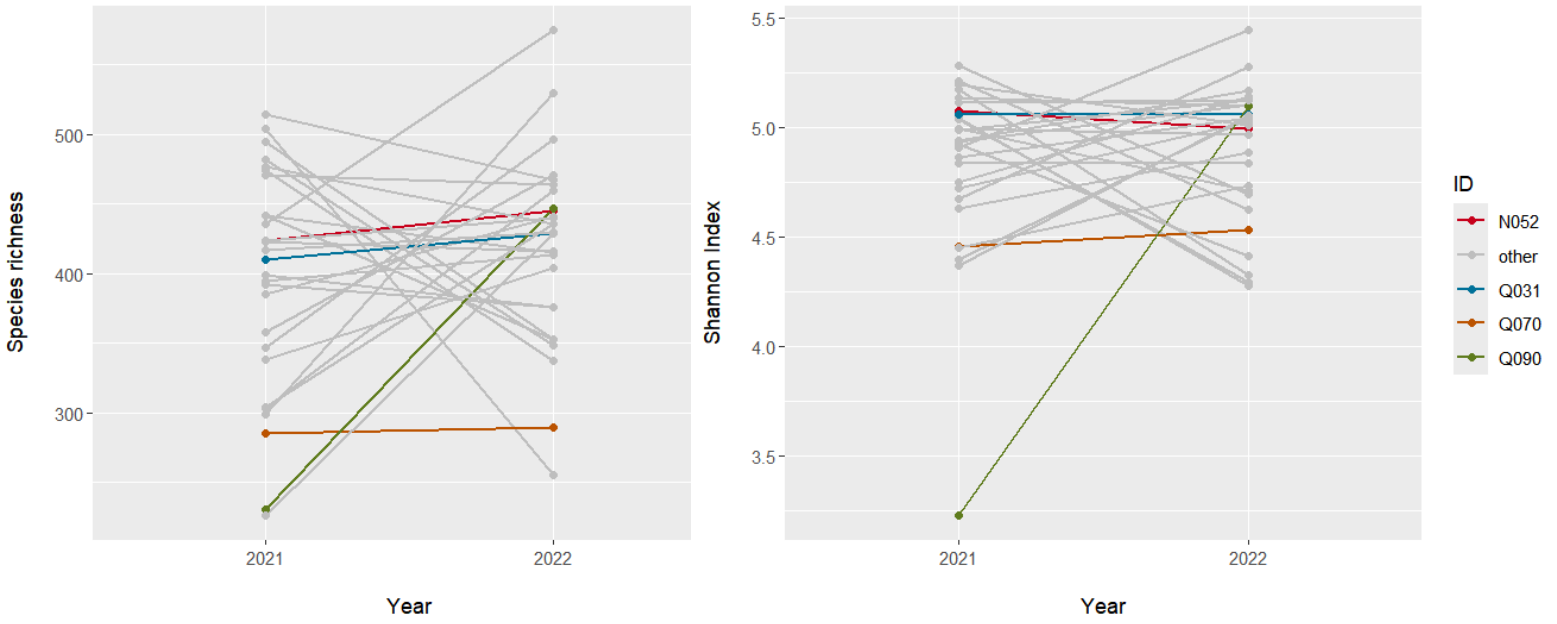

Figure S2.5 Inter-year variation (2021-2022) of the gut microbiota ASV richness and Shannon diversity index of eastern chipmunks (n = 28) of Mounts Sutton, Quebec, Canada. Four individuals were selected to do a comparative investigation between the composition, ASV richness, Shannon index and phylum relative abundance through time: Q031 (blue), N052 (red), R085 (orange) and Q090 (green).

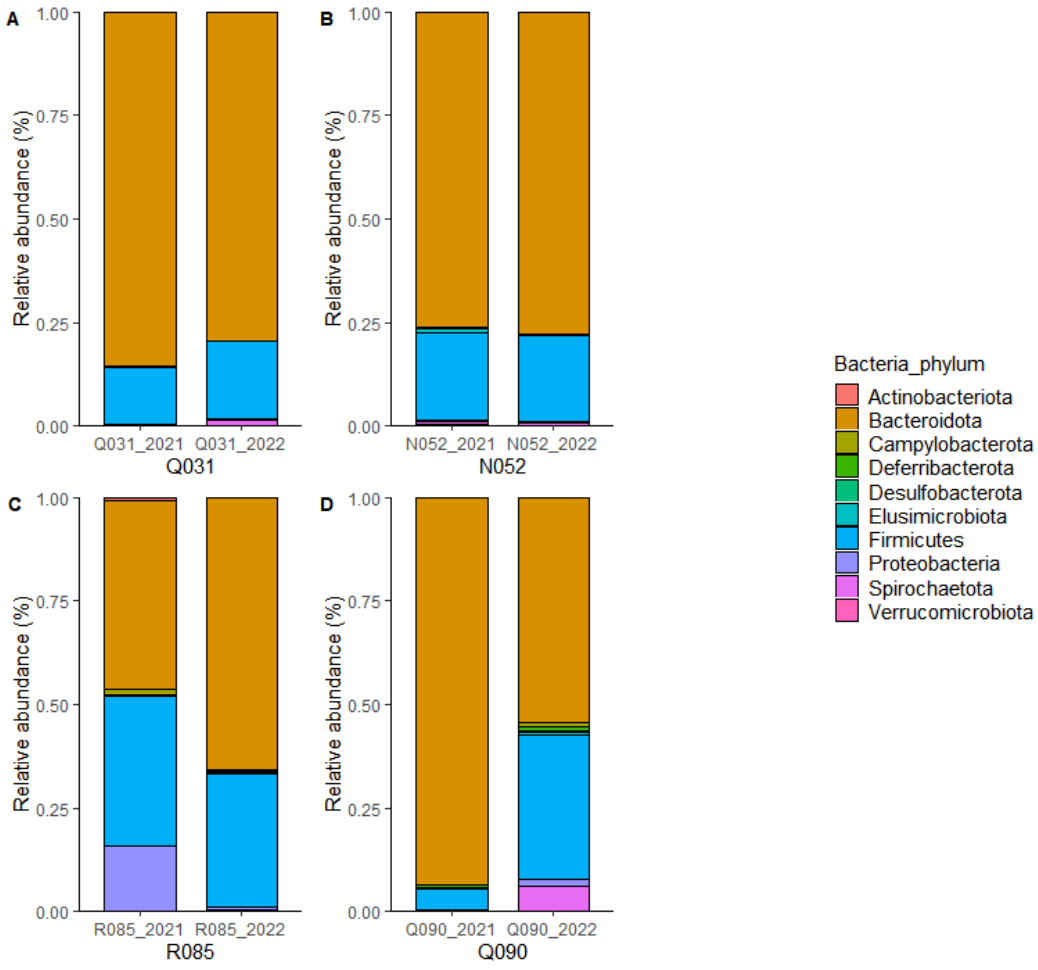

Figure S2.6 Inter-year variation (2021-2022) in bacterial phylum relative abundance of the gut microbiota of eastern chipmunks of Mounts Sutton, Quebec, Canada. Four individuals were selected to do a comparative investigation between the composition, ASV richness, Shannon index and phylum relative abundance through time: Q031, N052, R085 and Q090.

CHAPTER 3
CONCLUSION

Given the pivotal role that gut microbes play in shaping host physiology, cognition, and behaviour, it is increasingly clear that these microbial communities must be factored into ecological and evolutionary investigations. Yet, studies on wild animal microbiotas remain scarce compared to those on captive model species, leaving a gap in our understanding of how these communities function in natural environments. This thesis addressed this gap by examining the temporal dynamics of gut microbes in a wild chipmunk population and exploring the links between microbiota and host characteristics, with a particular focus on behaviour. To my knowledge, this is the first study to both partition the variance in gut microbe communities across multiple ecological levels and integrate animal personality theory into microbiome research. In this chapter, I revisit the key findings of my project, discuss their contributions to the field of research, address the limitations, and propose potential avenues for future studies.

3.1 Ecological implications and contributions

For the first objective, I explored the relationships between gut microbiota α-diversity (measured by the Shannon diversity index and ASV richness) and composition, and various host characteristics, including four behavioural traits. The findings confirmed that measurable behavioural traits are linked to gut microbiota composition, even in the wild, with minimal intervention to the animals' natural state. Specifically, chipmunks with a high exploration speed and those spending more time in the central area of the open-field exhibited different bacterial compositions compared to individuals that were trapped more frequently and visited a broader range of traps throughout the year. The divergence of microbial communities associated with behaviours measured in the open-field compared to those inferred from trapping data suggests that the former may reflect an individual's physiological state, while the latter may be more likely to reflect microbial transmission from environmental sources. These findings not only reveal new dimensions of how behavioural traits covary with gut microbiota but also suggest potential pathways through which gut microbes may influence or be influenced by host behaviour. I believe that the behavioural component is one of the strongest aspects of my research project, as there are still very few studies that have incorporated animal personality into microbiome research.

Age emerged as a significant predictor of both α-diversity measures, with juvenile chipmunks hosting less diverse microbial communities compared to adults. While these results align with expectations, gathering data on a larger spectrum of wild hosts is essential to draw generalized conclusions on host-microbiota

relationships. Additionally, the confirmation of these anticipated relationships validated the sampling methodology and confirmed that the age categories used in the long-term chipmunk study in southern Québec are appropriate for microbiome research.

Interestingly, sampling site, which was initially treated as a control variable, turned out to be a significant predictor of microbiota composition. This result broadened the environmental scope of the study, emphasizing the critical role of microhabitat in shaping gut microbiota. Since there is no significant genetic sub-structuring among chipmunks at different sites (Chambers & Garant, 2010), these differences in gut bacterial composition are likely driven by variations in resources and habitat structure. Future studies would benefit from integrating more detailed environmental data on the sampling sites, such as the abundance of tree and herbaceous species, the quantity of decaying wood, or soil humidity, to further elucidate the mechanisms of microbial transmission and their ecological significance.

For the second objective, I focused on the stability of the gut microbiota, or its variation within individuals throughout a trapping season. While Shannon diversity remained stable over time, chipmunks' gut microbiota exhibited different dynamics of ASV richness. Additionally, the composition of bacterial communities showed minimal variation at the within-individual level. Ecological theory posits that community stability is crucial for ecosystem health and productivity. When applied to microbiome research, a stable gut microbiota is deemed vital for host health, as it enables microbes to consistently perform their functions over time (Lozupone et al., 2012; Coyte et al., 2015). This study stands out as one of the few that investigate gut microbiota stability from an ecological perspective, taking multiple samples from the same individuals across different time points in their natural environment.

In the third and final objective, I investigated individuality, or among-individual differences, in the context of gut microbiota in wild chipmunks. Given the high variability typically observed among individuals' microbiomes, these differences have the potential to drive selection on host traits that are influenced by microbial communities (Moeller & Sanders, 2020). I was interested on the effect of individual identity on the microbiota both over a short timescale (one active season) and across a two-year period. My findings revealed that while gut microbiota α-diversity and composition were individualized over the short term, individual signature barely persisted when analyzed over a longer period. This aligns with the results of Risely et al. (2022), who found that host identity explained a large proportion of gut microbiota composition within a span of less than two months, but that the year of sampling became the dominant predictor over extended periods. Moreover, these results allow for speculation on the direction of causality between gut microbiota and behaviour within this population. Since behavioural differences in this population are known to be consistent over time and that the microbiota composition does not persist from year to year, it is unlikely that gut microbes are driving interindividual behavioural differences in this population. Finally, I

think that applying repeatability analyses, a well-established technique in ecology, to microbial data holds great potential for bridging microbiome research with evolutionary ecology.

3.2 Limitations

One of the primary limitations of this research project is its observational nature, making it challenging to determine the direction of causality. While correlations between gut microbiota and host traits provide valuable insights, future experimental studies or the application of advanced statistical models are needed to untangle the directionality of these relationships. Feedback mechanisms could also blur the impact of confounding variables or introduce time lags in the relationships between variables (Davidson et al., 2020). Additionally, the potential influence of unmeasured environmental or genetic factors cannot be entirely ruled out, further complicating causal interpretations. Structural equation modeling might offer insights into the direction of causality and help differentiate between direct and indirect effects, though it requires assumptions and priors that were not available for this study (Laughlin & Grace, 2019). Nonetheless, the descriptive data provided here lays a crucial foundation for future research aimed at testing the direction of these effects.

Another significant limitation of this study was the sampling methodology employed in 2021, which served as a pilot year for incorporating gut microbiome sampling into the Chipmunk Project. During this initial year, we collected one sample per individual in the spring and a second one in the fall. Unfortunately, many chipmunks captured in the spring were not recaptured later in the year, resulting in a single sample for most individuals. Only 14 chipmunks were sampled twice in 2021 and recaptured in 2022, complicating comparisons of microbial communities between the two years. As a result, the lack of repeated sampling in 2021 hindered our ability to assess inter-year stability and necessitated adjustments in our statistical methods for analyzing individuality, which made comparing intra- and inter-year results more challenging. Additionally, the two years offered contrasting ecological conditions, with one being a mast year and the other a non-mast year. More consistent sampling across both years would have allowed for a more robust comparison of how pulsed resources dynamics affect wild animal's gut microbiota.

I also attempted to incorporate a genetic component into this project, as it could have significantly enhanced our understanding of gut microbiota variation in the wild. By estimating heritability and assessing the extent of maternal microbial transmission, I hoped to unravel the genetic influences on microbial communities. However, despite the pedigree assessments that are integral to the Chipmunk Project, not enough mothers could be assigned to our sampled individuals to perform these types of analyses effectively.

3.3 Perspectives

Research on wild microbiomes has implication for understanding host health, population dynamics, community ecology, evolutionary theory and many other fields. This study highlights the complex relationships between host traits, environmental factors, and gut microbiota composition and α-diversity in wild eastern chipmunks. By detailing the structure of microbial communities across various ecological and temporal scales, it provides additional foundations for understanding microbiota dynamics in natural populations. This work also raises various questions and opens up several promising avenues for future research.

Investigations into microbiota-behaviour relationships in the wild would greatly benefit from integrating physiological data, such as fecal glucocorticoid concentrations, to measure stress responses. Glucocorticoids, key messengers of the HPA axis, have been linked to gut microbiota variation in wild red squirrels (*Tamiasciurus hudsonicus*; Petrullo et al., 2022). The next step in bridging the gap between gut microbiota and behaviour in natural conditions, as well as understanding the broader ecological relevance of the microbiota-gut-brain axis, would be to incorporate all these measures into a single, cohesive study.

In further research, one should consider extending the temporal scope to multiple years when working on pulsed resource systems in order to encompass several periods with analogous ecological parameters. The conditions of these ecological systems provide unique opportunities to study the stability and resilience of gut microbial communities. By tracking these systems across multiple mast and non-mast years, researchers could gain deeper insights into how fluctuations in resource availability influence microbiota composition. Moreover, such long-term studies could reveal patterns of microbiome recovery or adaptation following environmental disturbances, providing a clearer understanding of the role that gut microbiota play in host fitness in dynamic environments.

Given the intricate relationships between the hosts and their microbiota, further research in wild populations is essential to uncover the mechanisms that influence individuality and stability of gut microbe communities, as well as the links between microbiota and host behaviour. Such studies will contribute to a more comprehensive understanding of the gut microbiota's role in natural ecosystems, shedding light on how these communities both influence and are influenced by their host and the environment.

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