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The Effects of Host Genotype and Maternal Transmission on the Gut Microbiome of Wild Wood Mice (*Apodemus sylvaticus*)

- Viewed Through the Lens of Ecological Theories

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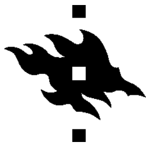

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Tiivistelmä – Referat – Abstract

The gut microbiome of mammals plays many important roles in the host, including preventing colonization of pathogens, maintaining intestinal homeostasis, helping digest nutrients and even affecting host behavior. The composition of mammalian gut microbiota varies greatly between individuals, species and in time. When a mammal is born, it acquires its first, mostly anaerobic, gut microbiota through maternal transmission in the birth canal. After the initial transmission of bacteria, host genotype, especially genes related to immunity, become an important factor that helps determine which species get to stay in the gut and prosper. In adulthood age, sex, diet, disease and contact with others all become important shapers of microbiome composition.

Since microbial communities are comparable to any macroecological communities, they can be explained through ecological theories. For example, community assembly theory can help distinguish the effects of input (e.g. transmission) from selective processes (e.g. filtering host genotype) on gut microbiome composition. Community assembly can lead to multiple stable equilibria determined by which species colonized the area first ("priority effect"), emphasizing the importance of early transmission, such as that maternal transmission birth. Metacommunity theory on the other hand, views a large ecosystem as a mosaic of patches and can be helpful in describing the composition of the microbiome in adult individuals.

In this thesis, I use community assembly theory and metacommunity theory as a framework to explore determinants of individual gut microbiome composition in wild European wood mice (*Apodemus sylvaticus*). Specifically, I set out to investigate how much of the gut microbial community variation was accountable for host relatedness and how much of this effect is due maternal transmission (input) versus host genotype (filtering). To find out more about what affects the composition of the gut microbiome in wild animals, I collected both tissue and microbiome samples from wood mice in the Wytham woods research area near Oxford, Great Britain. In addition to the data collected in Wytham, I was given another similarly collected dataset from Silwood Park. My study questions were: What proportion of gut microbiome composition in wood mice is determined by host genotype? Do mothers affect their offspring's microbiome more than fathers through maternal transmission of bacteria?

DNA extractions and mouse genotyping were done by me in the MES laboratory at the University of Helsinki. Sequencing of microbial DNA was done by my co-supervisor at Royal Veterinary College in London. Microbiome similarity was compared to host genetic relatedness using Mantel test and likelihood ratio tests on linear models with dyadic data (comparing relatedness and microbiome similarity of each pair).

According to the results, related individuals had a significantly more similar microbiome in Wytham, but not in Silwood. In both populations, microbiome similarity was also affected significantly by age and home range area. The general trend was, that mother-pup and fullsib pairs had more similar microbiome than unrelated pairs (though this effect was significant only in Wytham) and father-pup pairs had a more different microbiome than unrelated pairs (though this effect was significant only in Silwood). All data combined, mice had significantly more similar microbiome with their mother than father. The higher similarity between mother-pup pairs and full siblings can be explained by maternal transmission and postnatal physical contact. Since the father's effect is purely genetic, their microbiome differing from their offspring even more than from unrelated individuals could be explained by lack of physical contact and different age. Alternatively, females could even be choosing to mate with males with different immunogenotypes, and thus more different microbiome from themselves than expected by chance. Based on my results, transmission of bacteria during and shortly after birth is a key factor shaping microbiome composition and it might even account for the "genetic" effect seen in previous studies.

Avainsanat – Nyckelord – Keywords Host genotype, gut microbiome, maternal transmission, community assembly theory, metacommunity theory, priority effect, Wytham woods, Apodemus sylvaticus
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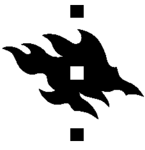

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Tiivistelmä – Referat – Abstract

Nisäkkäiden suolistomikrobiomilla on monia tärkeitä rooleja isännässä, joihin kuuluvat patogeenien kolonisaation estäminen, suoliston homeostaasin ylläpitäminen, ravinteiden sulattaminen ja jopa isännän käyttäytymiseen vaikuttaminen. Nisäkkäiden suolistomikrobiomin koostumus vaihtelee suuresti yksilöiden ja lajien välillä, sekä ajan kuluessa. Kun nisäkäs syntyy, se saa ensimmäisen, enimmäkseen anaerobisen, suolistomikrobiominsa maternaalitransmission kautta äidiltä synnytyksessä. Bakteerien ensimmäisen transmission jälkeen isännän genotyypistä, erityisesti immuniteettiin liittyvistä geeneistä, tulee tärkeä tekijä, joka auttaa määrittämään, mitkä lajit menestyvät suolistossa. Aikuistuessaan yksilön ikä, sukupuoli, ruokavalio, taudit ja fyysinen kontakti alkavat myös vaikuttaa mikrobiyhteisön koostumukseen.

Koska mikrobiomiyhteisö on verrattavissa suurempikokoisiin ekologisiin yhteisöihin, sen rakennetta voidaan selittää ekologisten teorioiden avulla. Esimerkiksi community assembly- teoria voi auttaa erottamaan bakteerien maternaalitransmission vaikutuksia isännän genotyypin vaikutuksista. Community assembly-teorian mukaan lajit, jotka kolonisoivat alueen ensimmäisenä vaikuttavat yhteisön lopulliseen rakenteeseen, kuten maternaalitransmission kautta suolistoon saapuvat bakteerit. Tätä varhaista kolonisaatiota korostavaa teoriaa kutsutaan myös nimellä ”priority effect”. Metacommunity-teorian mukaan taas ekosysteemit koostuvat erilaisista laikuista, jotka lisäävät diversiteettiä ja voivat auttaa kuvaamaan mikrobiomin koostumusta aikuisiällä.

Tässä pro gradussa käytän community assembly- ja metacommunity-teoriaa selittämään villien pikkumetsähiirten (Apodemus sylvaticus) suolistomikrobiomin koostumusta. Tutkin erityisesti sitä, vaikuttavatko äidiltä saadut bakteerit vai isännän genotyypin luoma ympäristö (suolisto) enemmän suolistomikrobiomin koostumukseen. Tutkimuskysymykseni ovat: Kuinka suuri osa pikkumetsähiirten suolistomikrobiomin koostumuksesta määräytyy isännän genotyypin mukaan? Vaikuttavatko äidit enemmän kuin isät jälkeläisten suolistomikrobiomiin bakteerien maternaalitransmission kautta? Lähdin vastaamaan tutkimuskysymyksiini keräämällä hiiriltä kudos- ja ulostenäytteitä Wytham Woodsin tutkimusalueelta Oxfordista, Iso-Britanniasta. Wythamista kerätyn aineiston lisäksi minulla oli käytössä vastaavanlainen aineisto toisesta hiiripopulaatiosta Silwood Parkista, Lontoosta. Suoritin DNA eristykset ja rakensin hiirille sukupuun Helsingin yliopiston MES-laboratoriossa alkuvuodesta 2018. Toinen ohjaajani sekvensoi mikrobien DNA:n Lontoossa Royal Veterinary Collegessa samoihin aikoihin. Suolismikrobiomin samankaltaisuutta verrattiin isäntäparien sukulaisuussuhteeseen Mantel-testillä ja ANOVA-testiä käytettiin vertaamaan lineaarisia malleja, joissa oli kontrollitekijöinä myös metadataa (vertasin äiti-poikas-, isä-poikas- ja täyssisarusparien mikrobiomin samankaltaisuutta).

Mantel-testin mukaan sukulaisilla oli samankaltaisempi mikrobiomikoostumus kuin ei-sukulaisilla, mutta tulos oli merkitsevä vain Wythamin populaatiossa. Molemmissa populaatiossa mikrobiomin samankaltaisuuteen vaikutti merkitsevästi myös ikä ja hiirten reviirien läheisyys. Yleisesti tulokset näyttivät, että äiti-poikas- ja täyssisaruspareilla oli enemmän samankaltainen mikrobiomi, kuin ei-sukulaisilla (vaikka tämä vaikutus oli merkitsevä vain Wythamissa) ja isä-poikaspareilla oli erilaisempi mikrobiomi, kuin kaikilla muilla pareilla (vaikka tämä vaikutus oli merkitsevä vain Silwoodissa). Kun aineistot yhdistettiin, hiirillä oli merkitsevästi samankaltaisempi mikrobiomi äitinsä, kuin isänsä kanssa. Äiti-poikas- ja täyssisarusparien samankaltaisuus voidaan selittää maternaalitransmission ja synnytyksen jälkeisen fyysisen kontaktin avulla. Koska isän vaikutus poikaseen on puhtaasti geneettinen, ero voisi selittyä eri iän ja fyysisen kontaktin puutteen avulla. Vaihtoehtoisesti on mahdollista, että naaraat valitsevat tietoisesti parittelukumppanin, jolla on erilainen immunogenotyyppi ja siten erilainen mikrobiomi kuin sattumanvaraisesti olisi odotettavissa. Tulosteni perusteella bakteerien maternaalitransmissio synnytyksen aikana ja pian sen jälkeen ovat keskeinen mikrobiomin koostumusta määrittävä tekijä ja se mahdollisesti selittää myös aiemmissa tutkimuksissa havaittua geneettistä vaikutusta.

Avainsanat – Nyckelord – Keywords

isännän genotyyppi, suolistomikrobiomi, maternaalitransmissio, community assembly-teoria, metacommunity-teoria, priority effect, Wytham woods, Apodemus sylvaticus

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Contents

1. Introduction 1
1.1. The gut microbiome of mammals 1
1.2. What defines the composition of the gut microbiome in mammals? 2
1.3. Correlative and experimental evidence on maternal and genetic effects on the microbiome 4
1.4. Ecological theories explaining microbiome composition 5
Ecological theories in macroecological communities 7
Ecological theories and the microbiome 8
1.5. The objectives of this thesis 9
1.6. Why is it important to study host genotype and microbiome composition? 10
1.7. Study species and study site 11
2. Materials and methods 12
2.1. Collection of tissue and fecal samples 12
2.2. DNA-extraction and mouse genotyping 13
Pedigree reconstruction 15
2.3. Sequencing and pre-processing microbial DNA 16
2.4. Statistical analyses 17
Making matrices 17
Gut microbial composition and relatedness 18
Controlling for other factors 19
Does the type of relationship matter? 20
3. Results 21
3.1. General description of wood mouse population genetics and microbiome 21
3.2. Gut microbial composition and relatedness 24
3.3. Effect of other co-variates 24
3.4. Does the type of relationship matter? 26
4. Discussion 30
4.1. Differences between the populations 30
Bray-Curtis vs. Jaccard 30
4.2. Gut microbial composition and relatedness 31
4.3. Drivers of gut microbial composition 33
Wytham 33
Silwood 33
4.4. Does the type of relationship matter? 34
Mother knows best 34
Fathers and siblings 35
4.5. Future directions 36
5. Conclusions 37
6. Acknowledgments 38
7. References 38
8. Supplementary data 47

1. Introduction

1.1. The gut microbiome of mammals

Mammals, among other animals, are hologenomic, meaning they consist not only of their own genes but of the genes of their symbiotic microbes and together they can be seen as ecological or even evolutionary units (Ley et al. 2008; Zilber-Rosenberg & Rosenberg 2008). Mammals have never existed without their microbiome and microbial cells easily outnumber mammal cells in an individual (Foster et al. 2017). Especially the gut microbiome has been the subject of great interest during the past years. It consists of both strict and facultative anaerobic bacteria, archaea, viruses, and eukaryotic microbes, of which bacteria are most dominant and have been studied the most (Bäckhed et al. 2005). Although the gut microbiome is vital to its host, connecting host physiology to its broader ecosystem, most research on bacteria in our gut in the past has been done in humans and laboratory animals (such as rats and mice) and has focused primarily on single species of pathogens. Therefore, there are significantly less data on the mutualistic gut microbiota of wild animals.

The gut microbiome can expand the host's physiological potential and facilitate ecological adaptation (Goodrich et al. 2016a) by preventing colonization of pathogens (Reviewed by Hooper et al. 2012), maintaining intestinal homeostasis (Rakoff-Nahoum et al. 2004) and helping digest food and produce nutrients (Mackie 2002). For example, the rumen of ruminants is rich in mutualistic bacteria that enable the fermentation of cellulose from food material (Mackie 2002). Gut bacteria are also of key importance to host immunity (Round & Mazmanian 2009) and even chemical (scent) communication (Theis et al. 2013). Additionally, the gut microbiota can have a direct effect on the behavior of an animal through the Microbiome-Gut-Brain axis or the vagus nerve (Reviewed by Montiel-Castro et al. 2013). This complex link between the gut microbiome and the brain is bi-directional, with the brain ensuring maintenance of gastrointestinal functions and the gut responding by producing neuroactive molecules that activate the vagus nerve and affect the mood and higher cognitive functions of the host (Bravo et al. 2011; Foster et al. 2017).

It is clear that the gut microbiome is of crucial importance to its host and it has even been called "the forgotten organ" (O'Hara & Shanahan 2006). But unlike other organs, the microbiome is also a complex ecological community, that is affected by the same factors that affect any other ecological community. Aspects that matter to the host are: which taxa are present, how abundant they are and how stable the community is. For example, some taxa, such as the well-known genera *Lactobacillus* have direct beneficial effects on host metabolism (Bennet & Nord 1987). Furthermore, the more emergent properties of the microbial community, such as diversity or

stability are important to host immunity, as they affect the likelihood of pathogen invasion (Lin et al. 2013). Overall, the host's health is affected in either a positive, negative or neutral way depending on the composition of the gut microbiome and how compatible it is with the host.

Since the composition of the gut microbial community matters to the host, it is important to understand what forces shape the microbiome. In 2012 the Human Microbiome Project Consortium established that the human microbiome is both extremely diverse and that it varies greatly between individuals. Related individuals, however, have been found to have more similar gut microbiome composition, than unrelated individuals, suggesting host genotype has an impact on the microbial taxa residing in the gut (Turnbaugh et al. 2009; Yatsunenko et al. 2012; Goodrich et al. 2014). This makes sense since several studies have found that host genotype has an impact on the gut microbiome in a diseased state (e.g. Tysk et al. 1988; Fouts et al. 2012; Karkman et al. 2017), giving reason to believe that this is true in a healthy individual as well. The composition of the microbiome can also change because of diet (Maurice et al. 2015) or over time, especially during illness or if the host's body is still developing (Bäckhed et al. 2015). With age, however, the microbiome seems to stabilize and studies supporting a somewhat stable temporal microbiome have emerged during the past few years in both human and murine models (Friswell et al. 2010; Lozupone et al. 2012). While mammalian species vary in the composition (Human Microbiome Project Consortium 2012) and temporal stability of their microbiome to some extent (Sonoyama et al. 2009), the above general assumptions most likely apply to most mammalian species.

1.2. What defines the composition of the gut microbiome in mammals?

Many phenotypic traits can be defined by an individual's early life, a fact that applies to the microbiome as well (Burdge et al. 2008; Raulo 2015). A mother's womb is considered to be a sterile place and the infant residing inside is germ-free (Favier et al. 2002), unless the mother has an infection (Jimenéz et al. 2008; but also see critique on sterile womb paradigm: Funkhouser & Bordenstein 2013). When the baby is born, it acquires an optimized combination of its first, mostly anaerobic, gut microbiota through maternal transmission in the birth canal (Mackie et al. 1999). Bacteria belonging to the *Bifidobacterium* and *Lactobacillus* genus are among the most common and important bacteria transmitted. They are known to help the infant digest milk and hinder pathogen colonization (Bennet & Nord 1987; Dominguez-Bello et al. 2010). Natural birth is an important shaper of microbiome composition, which is evident from studies of abnormal birth: If the baby is born through Cesarean section, its gut microbiota resembles that of the mother's skin and oral microbial community instead (Biasucci et al. 2008; Dominguez-Bello et al. 2010) and can remain lower in species diversity for years (Biasucci et al. 2008; Karkman et al. 2017). This

reduced diversity can cause a myriad of health issues with symptoms lasting into adulthood, such as asthma, allergies and even autism (Bager et al. 2008; Penders et al. 2006; Rosenfield 2015). These studies show that the initial acquirement of microbiota has an essential effect on the consequent community assembly and development of microbiota within the gut of an individual.

Another important and often overlooked factor shaping the microbiome is host genotype: Various genetically determined aspects of host immunity and physiology, such as the immune and endocrine systems, can be thought to function as a selective filter, that determines which species get to stay in the gut and prosper (Turnbaugh et al. 2009; Bolnick et al. 2014; Kubinak et al. 2015) In recent years, these common themes have been found in both mouse and human models (Reviewed by Goodrich et al. 2016a). For example, dysbiosis or microbial imbalance of the gut in humans has been associated with several gastrointestinal illnesses, such as Crohn's disease. Crohn's disease, in turn, is heritable and linked to mutations in numerous genes, which affect the composition of the gut microbiome (Tysk et al. 1988). Another example involves an immune-related protein, the major histocompatibility complex (MHC), which is an essential part of the acquired immune system with a high degree of polymorphism (Toivanen et al. 2001; Lanyon et al. 2007). A study by Toivanen et al. (2001) found that inbred lines of mice that differed only in MHC-genotype, still had significantly different gut microbiomes, providing strong support that MHC-genotype is a realistic driver of microbiome composition. In addition, one study has even found that a mutation in a single host gene can lead to specific restructuring of commensal gut microbiota (Khachatryan et al. 2008).

To support this notion, studies have found a core of over 50 taxa in the microbiome of human subjects (Tap et al. 2009; Qin et al. 2010, also see Turnbaugh et al. 2009). A similar observation has been made in a large murine population under controlled conditions as well (Benson et al. in 2010): QTL (Quantitative trait loci) analysis was used to detect 18 mostly immuno-related loci, which were significantly associated with the abundances of taxa and were subject to host genetic control. This supports the idea of a genetically determined "core microbiome" in vertebrate hosts (Benson et al. 2010). Some authors have even proposed classifying human gut microbiomes into different enterotypes, or categories of core gut microbiota based on microbial composition, to aid development of personalized medical care (Costea et al. 2018).

In addition to host genotype, early development (the first few weeks or even years depending on the species) of the offspring's life is crucial to the succession of the gut microbiome (e.g. Koenig et al. 2011; Jin et al. 2011). In most mammals, the offspring is mostly taken care of by the mother (Clutton-Brock 1991), which means the mother's diet and nest conditions affect the composition of the offspring's microbial community the most at first. Since mammals nurse their

young, the constant physical contact and feeding work as a continuous transmission of bacteria from and between the mother and the offspring (Martin et al. 2009; Jin et al. 2011). In cases where future sustenance is particularly demanding to digest, like eucalyptus for koalas (*Phascolarctos cinereus*), the mother may even feed its pup fecal material to help it receive the bacteria needed for digestion (Osawa et al. 1993). The full effect of the mother on the microbiome (maternal transmission during and after birth as well as the genotype inherited from the mother) is most prominent during these early stages but is reduced after weaning (Wolf & Wade 2009).

When the offspring finally leaves the mother, other factors start to affect the individual's microbiome, such as the environment, diet, sex (hormones), disease, age, and social contact with others. For example, wood mouse (*Apodemus sylvaticus*) microbiota has been found to fluctuate paralleling seasonal diet shifts from seeds to insects (Maurice et al. 2015). Changes in diet have also been reported to affect the microbiome differently depending on the sex of both three-spined sticklebacks (*Gasterosteus aculeatus*) and laboratory mice (Bolnick et al. 2014). Similar to dietary shifts, the microbiome is known to vary between disease states. For example, as a response to acute liver disease, the amount of *Firmicutes* and *Actinobacteria* have been noticed to increase in male wild-type BALB/c mice, suggesting disease affects gut microbiome composition (Fouts et al. 2012). In addition, the amount of circulating cytokines (proteins important in immune responses) have been found to correlate with the gut microbiome composition of old mice (Conley et al. 2016). Furthermore, the gut microbiota of social group-living animals, such as chimpanzees (*Pan troglodytes*), red-bellied lemurs (*Eulemur rubriventer*) and yellow baboons (*Papio cynocephalus)* has been found to be more similar among group members, than with foreign individuals (Degnan et al. 2012; Raulo 2015; Tung et al. 2015). Although environmental factors play a key role in shaping the microbiome, the early determinants, mainly genotype and maternal transmission have a constant underlying impact on the composition of the microbiome throughout the host's life.

1.3. Correlative and experimental evidence on maternal and genetic effects on the microbiome

Both correlative and experimental studies have been done to prove the effects of early determinants such as maternal transmission on the gut microbiome. A recent study on North American red squirrels (*Tamiasciurus hudsonicus*) found that adult offspring had a significantly more similar gut microbiome with their mother than with their father or unrelated individuals. This not only indicates that gut microbiota can be maternally transmitted, but also that the effects of maternal transmission persevere until adulthood (Ren et al. 2017). Maternal transmission was also found to explain 26 % of variation in the gut microbiome of laboratory mice, according to Benson et al. (2010).

To experimentally test how much maternal transmission influences the gut microbiome in laboratory mice, embryos from two different mouse strains were implanted into a mother belonging to yet another distinct strain. The results showed that mouse pups from different strains that were born together shared a similar microbiome, suggesting that maternal transmission does, in fact, have a significant effect on gut microbiome composition (Friswell et al. 2010). However, it is important to note, that not all studies have found a significant impact by maternal transmission. For instance, one study using DNA fingerprinting methods showed that microbiota differed significantly between different genotypes of laboratory mice and hardly any maternal effect was detected (Kovacs et al. 2011). This only shows, that more evidence is needed to corroborate the theories about the effects of maternal transmission.

Despite individuals often varying in gut microbial composition, family members have been observed to have more similar microbiomes compared to unrelated individuals. The host genotype impacts food preferences, gut physiology and characteristics of immunity, which all have potential in influencing community composition of the microbiome (Goodrich et al. 2016b). A few correlative studies have been conducted to determine how much host genotype affects the gut microbiome of humans. For instance, a study on monozygotic and dizygotic twins found that abundances of specific taxa were more highly correlated within monozygotic twins, suggesting that these taxa were more affected by host genotype than other bacterial species (Goodrich et al. 2014; but also see Turnbaugh et al. 2009). An updated version of the experiment found that up to 8.8 % of taxa had a heritability (h^2) greater than 0.2 and that the most heritable family of bacteria was *Christensenellaceae* with $h^2 = 0.39$ (Goodrich et al. 2016b). Additionally, a previously mentioned study by Kovacs et al. (2011) observed, that genotype had a stronger influence than sex, on the gut microbiota of laboratory mice. While experimental studies on the effect of maternal transmission have been attempted before, there are currently no experimental (selective breeding) studies investigating the effects of genotype on the gut microbiome.

1.4. Ecological theories explaining microbiome composition

During the past few years, there has been a growing interest in the use of ecological theories to explain the composition of the gut microbiome (Costello et al. 2012). After it was discovered, that the microbiome varies within healthy individuals as well as diseased ones (Palmer et al. 2007; Ravel et al. 2011), the importance of recognizing the processes behind the variation was emphasized. Ecological theories strive to explain phenomena that transpire in communities of organisms, such as spatial and temporal patterns of species diversity, which occur in microbial communities as well (Costello et al. 2012). Dispersal, selective filtering, speciation, and extinction all affect a community,

whether it is on a macro or micro scale. The gut microbiome is more than just a mixture of species that have happened to colonize the intestine of an organism. Like in a macroecological community, interactions between different species and the environment (in this case the host) all affect the composition of the community. Therefore, comparing the microbial community to a macroecological community may help determine the factors defining the composition of the gut microbiome (Costello et al. 2012; also see Karkman et al. 2017).

One theory that can be useful in explaining the composition of the microbiome is community assembly theory (Diamond 1975), which helps understand the processes determining species diversity in an ecosystem. Community assembly theory predicts either one stable equilibrium determined by species interactions and the environment ("environmental filtering") or multiple stable equilibria determined by which species colonized the area first ("priority effect"). In the former scenario, communities with similar environmental conditions should have similar composition, whereas in the latter scenario communities with similar environments may vary tremendously in composition (Diamond 1975; Chase 2003) based on initial input of species. This priority effect has been found to not only affect community structure, but also other properties of ecosystems, such as decomposition, productivity, and energy flow (Dickie et al. 2012). The process of community assembly is highly relevant for distinguishing the effect of maternal transmission from host genotype on microbiome composition: The former is a priority effect –scenario, whereas the latter is an environmental filtering-scenario.

Another theory useful in explaining variation in the microbiome is the metacommunity theory (Wilson 1992), which views a large ecosystem as a mosaic of patches. A metacommunity is considered to be a set of local communities that are linked by dispersal and that differ in species and their complex interactions, even if the patches are identical (Hanski & Gilpin 1991; Wilson 1992). Similar to priority effect, initial conditions affect the composition of the patch and the species coexisting in the whole metacommunity can surpass the number of species living in a single patch. This has also been found in computer simulations, where the model patches are identical, the species have similar colonization abilities and stochastic effects do not exist (Wilson 1992). Metacommunity theory is a good framework for microbiome community dynamics because host individuals can be seen as patches and the transmission of bacteria as dispersal.

Metacommunity theory consists of four different approaches that are referred to as the patch-dynamic, species-sorting, mass-effect and neutral perspectives. The patch-dynamic perspective views patches (e.g. individuals) as identical and assumes they can be either occupied or unoccupied by a selection of species that are affected by local dispersal, extinction, and colonization. The species-sorting perspective highlights the effects of patch quality and abiotic

features (or "host physiology features" in a microbiome) on the composition of the community, along with dispersal (Leibold et al. 2004). The mass-effect perspective emphasizes the effects of immigration and emigration on community composition and can be compared to the term "source-sink" used in metapopulation ecology. This means a high-quality patch may act as a constant source of species/individuals to a low-quality patch, which may otherwise not be able to sustain a population (Pulliam 1988; Leibold et al. 2004). And finally, the neutral perspective states that species are equivalent in their competitive and dispersal abilities, and community composition is determined by random demographic processes and limitation of dispersal (Hubbell 2001; Leibold et al. 2004).

Ecological theories in macroecological communities

Although community assembly theory remains controversial in the scientific community (e.g. Gotelli & McCabe 2002), several experiments and observations have proven both outcomes (single and multiple equilibria) to be possible (Chase 2003). For example, one study concluded that plants form deterministic communities that reach one stable equilibrium depending on the environment (Clements 1938). Another study showed that experimental removal of pioneer species after landslides in a Puerto Rican forest change the final composition of the forest (Walker et al. 2010), suggesting that priority effect does, in fact, impact the final composition. Furthermore, a study on phytoplankton found that colonization order was significantly influential when immigration rates are low (Robinson & Edgemon 1988), indicating that community assembly theory is useful in explaining the initial composition of an ecological community.

Studies on real-world metacommunities have also demonstrated that all parts of metacommunity theory can be used to describe community composition across a variety of ecosystems. For example, a study on the Glanville fritillary butterfly (*Melitaea cinxia*) and its parasite (*Hyposoter horticola*) showed that this patchily distributed host could escape the parasite to further-away patches, even when the parasite was abundant in near-by patches (Van Nouhuys & Hanski 2002). This is consistent with the patch-dynamic perspective. Similarly, coral reefs have been found to consist of different metacommunities of species due to varying productivity of the corals, upwelling of nutrients by water currents and disturbance, which can be explained by the species-sorting perspective (Cornell & Karlson 2000). Furthermore, a study on microarthropods living in a fragmented ecosystem of epilithic moss showed that providing ecological corridors between patches reduces loss of diversity, which can be understood through the mass-effect perspective (Gonzalez et al. 1998). And finally, a study on several competing damselfly species (*Enallagma* sp.) found that species do not differ significantly in fitness between different patches

(McPeek & Brown 2000), meaning the neutral perspective may be an effective explanation for this phenomenon.

Ecological theories and the microbiome

At the beginning of an individual's life the gut microbiome is an ecological community, in which variation can be explained through ecological processes on 3 levels (Figure 1). The first level is the transmission of bacteria to the gut at birth, the second level is the genetic makeup of the individual that determines which of the transmitted species can thrive and the third level is the different relationships between the species in the gut (competition, mutualism, predator-prey, etc.). To understand the adult composition of the gut microbiome, all levels should be considered. Ecological theories such as community assembly and metacommunity theory provide tools to understand these processes (Costello et al. 2012; Coyte et al. 2015).

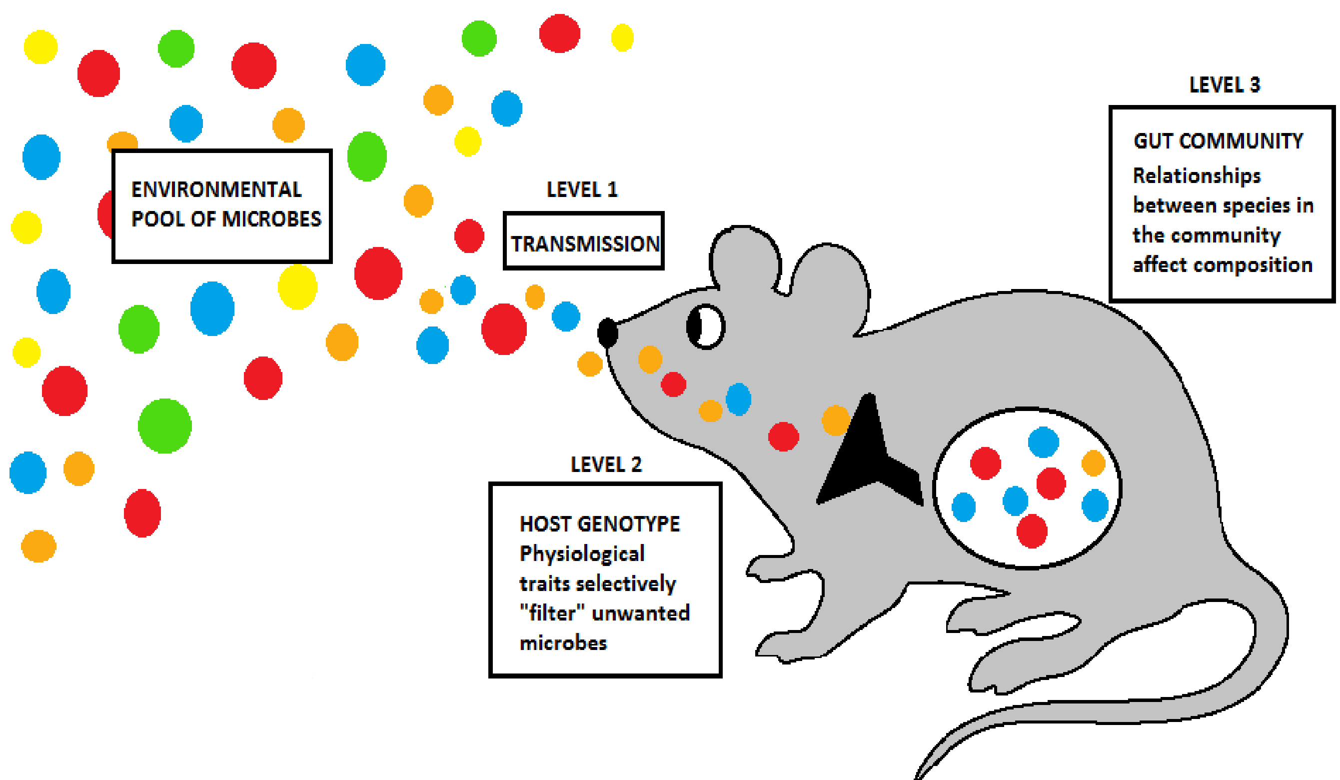

Figure 1. The 3 levels explaining gut microbial composition in mammals

When the offspring of a wild animal is born, it receives its first gut microbiota from its mother through the birth canal. These important and mostly anaerobic maternally transmitted bacteria establish a community in the gut and thus according to "priority effect" influence the outcome of the adult microbiome of the individual. If, however, there are complications during birth and the offspring is born through Cesarean section, different bacteria arrive in the gut first, resulting

in a completely dissimilar microbial composition in both child- and adulthood. Maternal transmission of bacteria that explains variation between related hosts or hosts living in the same nest/population can, therefore, be better understood through community assembly theory (Costello et al. 2012).

The microbiome of an adult individual is exposed to multiple different factors, which affect it as though it were a macroecological patch or an island. The gut community is shaped by dispersal, selection, and drift, just like any other ecological community. For example, monozygotic twins have identical DNA but still differ in microbiome composition (Turnbaugh et al. 2009), which can be explained by the patch-dynamic perspective. Then again, some bacteria may thrive in one host, but become extinct in another, because of "environmental" differences in the gut due to genetically determined aspects of host physiology, as stated in the species-sorting perspective. In addition to this, hosts living in tight-knit social groups, such as Verreaux's sifaka (*Propithecus verreauxi*) have similar microbiome compositions, because of the constant immigration of bacteria between individuals (Perofsky et al. 2017). This can be explained by the mass-effect perspective. And finally, some bacteria, such as *Bacteroidetes,* are very prominent in the human gut and their thriving does not seem dependent on host genotype, which can be explained by the neutral perspective (Costello et al. 2009).

Using these ecological theories to explain the composition of the microbiome is especially useful because maternal transmission and genetics can be seen as processes happening on two different ecological levels (maternal transmission happening on the first level and genetic effect on the second) and therefore should be explained by theories that consider these levels. Gut bacteria have different ecological niches, which means their abundance and effect on the host are affected by both levels in different ways. For instance, *Bifidobacterium* and *Lactobacillus* are mostly maternally transmitted bacteria (first level), that affect the host's central nervous system and thus affect their behavior indirectly. The mutualistic effects of these bacteria also include reducing anxiety and depression-related behavior (Bravo et al. 2011; Raulo 2015). Despite these maternally transmitted bacteria being important, the genetic makeup (second level) of the host eventually affects how well they can survive in the gut, which may or may not alter host behavior.

1.5. The objectives of this thesis

To find out more about what affects the composition of the gut microbiome in wild animals, I collected both tissue and microbiome samples from European wood mice (*Apodemus sylvaticus*) in the Wytham woods research area near Oxford, Great Britain. Using these samples, I investigated

the effect of host relatedness on the composition of the gut microbiome, and whether maternal transmission or host genotype play a bigger role in shaping the microbiome. In other words, the aim was to find out whether an individual's microbiome is more composed by their mother (affecting the microbiome through transmission and genotype) than their father (affecting the microbiome only through genotype). I attempted to explain the composition of the gut microbiome using ecological theories, such as community assembly (Diamond 1975) and metacommunity theory (Wilson 1992). The main questions I was interested in answering were:

1. What proportion of gut microbiome composition in wood mice is determined by host genotype?
2. Do mothers affect their offspring's microbiome more than fathers through maternal transmission of bacteria?

Based on previous literature I hypothesized that related individuals (e.g. parents and their offspring and siblings) would have more similar gut microbiomes compared to each other than to distantly related or unrelated individuals. I also predicted that offspring would share a more similar gut microbiome with their mother than father, because of the combined effects of host genotype and maternal transmission of bacteria. Furthermore, I expected that the effects of host genotype could be differentiated from the effects of maternal transmission, because females raise their young without paternal care, making the effect of the father purely genetic (Flowerdew & Tattersall 2008; Ren et al. 2017). The effects can be distinguished by comparing the offspring's microbiome to the father's and subtracting that effect from the similarity to the mother's microbiome. Throughout all this, my aim was to explain the composition of the gut microbiome using the above-mentioned community assembly and metacommunity theory framework.

1.6. Why is it important to study host genotype and microbiome composition?

A substantial amount of correlative evidence can be found on the importance of both maternal transmission and host genotype on the gut microbiome of animals. However, this evidence is mostly derived from controlled facilities such as laboratories, which differ from the conditions wild animals live in immensely. Since it is easier to control for factors affecting the microbiome (such as diet and pathogens) in laboratories, this seems to be the easiest approach to studying the microbiome. Nevertheless, the microbiome is a trait that essentially spans across multiple hosts and their living environments, and thus to be able to understand how the microbiome is composed, wild animal studies are needed.

The more is known about the gut microbiome and its composition, the better we will understand how it functions and what the ultimate effects on the host are. Strongly heritable bacteria

could have profound consequences on the ecology and evolution of all mammals and it is crucial to understand the deterministic and foreseeable interactions between hosts and their microbiomes. The genetic analysis of micro-communities fuses the fields of ecology and evolution to microbiology and offers a way to look beyond phenotype to study links at higher interspecies levels (Opstal & Bordenstein 2015). In addition, the results of microbiome studies can be utilized in other fields of science as well, such as developing medicine (Reviewed by Kuntz & Gilbert 2017), improving nutritional benefits of food (Kau et al. 2011) and species conservation of captive animals (Reviewed by Bahrndorff et al. 2016).

1.7. Study species and study site

My study species, the European wood mouse (*Apodemus sylvaticus*), is a common rodent found in most of Europe, which typically inhabits woodlands and fields. Wood mice are nocturnal and mainly feed on grain and seeds, although they can quite easily change their diet to invertebrates or fruit when seeds are not available. The wood mouse breeding season ranges from March to October (sometimes even longer) and they can have multiple litters per season. During breeding season females usually live in solitary nests, however, the home ranges of individuals may overlap. Wood mice are by no means monogamous and multiple paternity in litters has been recorded on several occasions. Pups are weaned at about 18 days of age and females raise their pups without paternal care (Flowerdew & Tattersall 2008).

I collected my data in Wytham woods, a temperate woodland owned by the University of Oxford near the John Krebs field station (5 kilometers from Oxford). The mean ambient temperature in Wytham ranges from 5 °C in January to 22 °C in July. The trapping site consists of open woodland areas and thick bramble bushes and the woods are dominated by ash (*Fraxinus excelsior*), sycamore (*Acer pseudoplatanus*) and blackberry brambles (*Rubus fruticosus*, Savill et al. 2011). The original trapping grid used for the study was 1 hectare in size, but we expanded it to 2.4 hectares during my research visit. Tissue samples were taken from each newly trapped wood mouse, along with fecal samples (for microbiome analysis) that were collected directly from the traps. Our traps also caught other small rodents inhabiting the area, such as yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Myodes glareolus*), but I did not use data from these captures in my thesis.

In addition to the data I helped collect in the fall of 2017, I used data collected previously by others from the same trapping grid starting from March 2015. I also used another dataset from a similar trapping site at Silwood Park, Berkshire (belonging to Imperial College London) collected between November 2013 and November 2015. The trapping site at Silwood Park contained an array

of different microclimates, varying from open woodland to patches of bamboo (*Sasa palmate)* and rhododendron (*Rhododendron ponticum*). Both the sample collecting methods and trapping grid size (2.4 hectares) were the same as in Wytham (although the trapping grid was smaller in Wytham at first).

2. Materials and methods

2.1. Collection of tissue and fecal samples

I participated in the collection of data from mid-September to the beginning of December 2017. Trapping was conducted approximately every other week (weather permitting) in the trapping grid, which was divided into 240 cells (100 m² each). We distributed 120 traps in a checkerboard manner in every other cell and trapping cells were alternated every trapping week. Small Sherman traps (LFATDG Folding Trap) baited with 6 peanuts, a slice of apple (Pink Lady®) and bedding material were used to trap the animals safely with minimal harm. Sherman traps contain a trigger platform that closes the trap door once an animal goes in, allowing only one individual to be trapped at a time. Traps were set out at dusk and collected at dawn to minimize the time captured animals had to spend in the trap

After the traps were collected, they were taken to nearby indoor facilities ("The Chalet") for animal processing. For each animal, we recorded the species, sex, age (juvenile, sub-adult or adult) and reproductive status (for females: whether they were pregnant, imperforate or perforate, and whether they showed signs of lactation or prominent nipples; for males: the extent to which their testes protruded from the abdomen – testes abdominal, small or large). Females were scored as reproductively active when they were either pregnant, perforate, lactating or had prominent nipples, and males when their testes were scored as small or large. Visible ectoparasites (ticks, fleas, mites) were counted, and right foot length and anogenital distance were measured three times with a dial caliper. For all new captures, a PIT tag (Francis Scientific Instruments) was inserted subcutaneously to provide identification, and all animals were scanned for a PIT tag at every capture. Ear tissue samples were taken from each newly caught animal using a 1.5 mm diameter ear punch (Vet-Tech Solutions Ltd.), with the ear punch pattern serving as a secondary means of identification in the case of PIT tag loss. Tissue samples were stored in 90 % ethanol and kept at -20 °C at the Royal Veterinary College, until further processing.

After all mice were processed, fecal samples (approximately 250 mg) were collected from each trap and placed in Eppendorf tubes. The sample tubes were labeled with the PIT tag number of

the individual the sample came from so that microbiome samples could later be connected to the correct tissue samples. Recaptured animals had multiple microbiome samples taken from them. Fecal samples were taken to the Royal Veterinary College immediately after fieldwork and stored at -80 °C until further processing. After each trapping session, all traps were thoroughly sterilized by washing in a bleach solution, to ensure no cross-contamination among fecal samples.

2.2. DNA-extraction and mouse genotyping

Tissue samples from 138 mice with corresponding fecal samples were genotyped. Tissue samples were sent to Helsinki (MES-laboratory, University of Helsinki) in late January 2018, where I conducted DNA-extractions using the QIAamp DNA Micro Kit (Qiagen, Netherlands). Extractions were carried out according to the manufacturer's instructions, using the "Isolation of Genomic DNA from Tissues" protocol. One extraction negative control (H_20 extracted in the same way as ear tissue) was included in each batch of samples. The DNA concentration of the extractions was checked using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific™) and all samples were then diluted to a concentration of ~20 ng/µl using purified water (Milli-Q®, Millipore Corporation).

Twelve primer pairs targeting microsatellite markers were ordered from MetaBion International AG for genotyping (Table 1). Microsatellite markers are tandem repeats of 1-6 nucleotides that are found at a high frequency in most taxa. They have high mutation rates (on average 5×10^{-4} mutations per locus per generation), that produce the necessary allelic diversity for genetic studies. Microsatellites are often shorter than sequenced loci (100-300 bp vs. 500-1500 bp), so they can be amplified with PCR even if some DNA degradation of the samples has occurred. Microsatellites also eliminate the issue of cross-contamination by non-targeted organisms, since they are mostly species specific. (Schlötterer 2000; Selkoe & Toonen 2006).

Locus	No. Alleles	Size range	Successfully genotyped samples	Hobs	Hexp	HWE	Null allele	Scoring Error
Apfl_BF6	9	340-410	138	0.717	0.780	NS	0.0370	0.001
AS-7	22	80-140	138	0.783	0.862	NS	0.0449	0.000
AS-11	20	230-280	137	0.920	0.911	NS	-0.0067	0.001
As-12	23	220-270	138	0.862	0.942	NS	0.0426	0.062
As-20	20	120-170	138	0.775	0.913	NS	0.0802	0.003
As-34	22	150-210	138	0.862	0.876	NS	0.0056	0.001
CAM-13	3	180-200	138	0.391	0.361	NS	-0.0451	0.023
GACAA12A	7	230-260	136	0.765	0.723	NS	-0.0321	0.001
GCATD7S	11	170-230	138	0.855	0.852	NS	-0.0047	0.015
MSAf-8	26	160-230	138	0.935	0.900	NS	-0.0227	0.002
TNF-CA	20	100-150	138	0.906	0.889	NS	-0.0122	0.003

Table 1. Summary data for microsatellite loci, including observed (Hobs)and expected (Hexp) heterozygosity, deviation from Hardy-Weinberg Equilibrium (HWE, NS = non-significant), null allele and scoring error rate.

The markers I selected were specifically designed for wood mice and chosen based on two PhD theses (Godsall 2015; Wilson 2014). However, after checking the genotyping results with the program Cervus 3.0.7 (Kalinowski et al. 2007) the marker GACAB3A deviated significantly from Hardy-Weinberg equilibrium and was therefore removed from the data (leaving 11 markers). A summary of the remaining 11 loci is shown in Table 1, obtained using Cervus 3.0.7 and Micro-checker 2.2 (Oosterhout et al. 2004). Cervus is a software that uses likelihood and simulation for parental assignment using genetic markers. Micro-checker on the other hand, is a software that tests the genotyping of microsatellites and helps identify genotyping errors due to null alleles (nonfunctional copies of a gene caused by a genetic mutation) or allelic dropout (the non-amplification of one of the alleles present in a heterozygous sample).

Genotyping was performed using four multiplexes and one single PCR reaction, based on PCR product size range and primer annealing temperature (Table 2). All the forward primers of the markers were ordered with dyes FAM, HEX or TAMRA added to them, to distinguish markers from each other in the multiplexes. The markers had a concentration of 100 µM when they arrived, so they were diluted to a standard working concentration of 10 µM using purified water (Milli-Q®, Millipore Corporation).

Locus	Fluorescent label	Primer Concentration (µmol)	Multiplex	Annealing temperature	Size standard	Source
AS-7	FAM	0.1	A	50	ROX	Godsall 2015
MSAf-8	HEX	0.1	A	50	ROX	Godsall 2015
AS-11	TAMRA	0.1	A	50	ROX	Wilson 2014
As-20	FAM	0.1	B	57	ROX	Godsall 2015
GCATD7S	HEX	0.1	B	57	ROX	Godsall 2015
As-34	TAMRA	0.1	B	57	ROX	Godsall 2015
Apfl_BF6	FAM	0.1	C	57	ROX	Godsall 2015
GACAA12A	HEX	0.1	C	57	ROX	Godsall 2015
TNF-CA	TAMRA	0.1	C	57	ROX	Godsall 2015
As-12	FAM	0.1	D	Touchdown 56-53	ROX	Godsall 2015
CAM-13	HEX	0.1	D	Touchdown 56-53	ROX	Godsall 2015
GACAB3A	TAMRA	0.1	Singleplex	55	ROX	Wilson 2014

Table 2. Multiplex composition and PCR protocols for microsatellite loci.

After dilution, PCR was run on each multiplex to amplify the marker sequences. A universal multiplex cycling protocol (Qiagen, Netherlands) was used every time, however, each multiplex had a separate annealing temperature (Table 2). The cycle consisted of a denaturation step (95 °C for 15 minutes x 1 cycle), a primer annealing step (incubation at 95 °C for 30 seconds, the annealing temperature for 90 seconds and 72 °C for 1 minute x 30 cycles) and an extension step (60 °C for 10 minutes x 1 cycle). Each PCR also included a negative (H_20) control. Gel electrophoresis was used to visualize products on 2% agarose gels, and no contamination was observed in extraction or PCR controls. The PCR products were diluted with purified water based on how strong the bands were on the gel (for example 1:300 of PCR product to water ratio). To prepare the samples for genotyping, 2.5 µl of size standard Genescan 500 ROX (Applied Biosystems™) was added to 1 ml of deionized formamide (Applied Biosystems™) and vortexed thoroughly. After this, 9 µl of the solution and 1 µl of the diluted sample (PCR product) were added to a FrameStar® 96-well ABI plate (4titude®). The plate was sealed with a Semi-Automatic Sheet Heat Sealer (4titude®) and stored in the freezer (-20 °C) to await genotyping, which was done by laboratory technicians using the 3730 DNA Analyzer (Applied Biosystems™). PCR and genotyping were repeated for any failed samples, but there were 4 amplifications that did not work for a certain marker despite multiple repeats.

Allele scoring was conducted using GeneMapper® Software version 5 (Applied Biosystems™). GeneMapper® is a genotyping software package that provides DNA sizing and allele calls for electrophoresis-based genotyping systems. I first set the size range and the dye used for each marker (Godsall 2015; Wilson 2014) and created panels for each of the multiplexes (Table

2). I then added the genotyping results to the corresponding panels and went through every sample individually. I auto binned clear peaks first (< 1000 relative fluorescent units) and then compared repeated samples to each other to determine and bin the rest of the actual peaks. When all alleles were scored I exported the table from GeneMapper® for pedigree reconstruction.

Pedigree reconstruction

The pedigree was constructed using COLONY 2.0.6.5 (Wang 2004), a computer program for parental and sibship inference from genotype data. This software was chosen because it can perform analyses for polygamous species and it accommodates genotyping error. It divides the samples into family clusters, in which individuals are related via sibship or shared parentage. The likelihood of a cluster is calculated based on Mendelian inheritance rules (Jones & Wang 2010). The likelihood function for a family cluster with an arbitrary genetic structure is calculated in the following way:

$$L = \Pr[R] \sum_g Pr[G|g, R] \Pr[g| R],$$

where *Pr[R]* is the prior probability of *R*, *G* is a vector of observed genotypes for all members of the cluster and *g* is a vector of their unobserved underlying genotypes. All possible parental genotypic combinations are accounted for in the summation (Wang & Santure 2009).

Since wood mice are polygamous, dioicous, diploid and may inbreed I also used these settings in the program. I set the length of the run to high and the analysis method to Full-Likelihood (FL) with no known sibship prior. After this, I imported the genotypes for offspring (all samples), potential mothers (females, n=58) and potential fathers (males, n=70) into COLONY. The program was then run multiple times to get the most accurate full sibship and parent pair estimates. After the pedigree reconstruction, I compared the results with the trapping data collected with all samples to exclude impossible pairs (based on age and time trapped) and visualized the results using Pedigree Viewer (Kinghorn 2011) to double check for potential issues: none were found.

2.3. Sequencing and pre-processing microbial DNA

Microbial DNA from the Wytham fecal samples was extracted according to manufacturer's instructions by PhD student Kirsty Marsh in the CEEED laboratory (Royal Veterinary College) using the Zymo Quick-DNA Fecal/Soil Microbe 96 Kit. The same protocol was used for the Silwood samples as well, only the DNA was extracted by my co-supervisor Aura Raulo. After extraction, the V4 region of the bacterial 16S rRNA gene was amplified with PCR using primers N501R and N701R (Caporaso et al. 2012). Each batch of 96 extractions included one extraction control (H_20). To avoid potential technical effects on downstream microbial data, samples were randomized across batches in both extractions and PCRs. The PCR products were sent to the Centre

for Genomic Research in Liverpool for addition of Illumina indices, clean-up, size selection and paired-end 250bp sequencing on an Illumina® MiSeq machine.

Before being able to describe the microbial community structure, the sequence data had to be cleaned and clustered into taxonomically sensible units. This was done using the DADA2 sequence processing pipeline, which pre-processes raw DNA sequence reads into a bacterial taxa-per-sample form. The advantage of DADA2 compared to other pipelines is that it distinguishes biologically meaningful variation from sequencing errors by building a case-specific error model for sequence quality (Callahan et al. 2016).

After processing the samples with DADA2, the 'phyloseq' package (McMurdie & Holmes 2013) in R (R version 3.5.1," Feather Spray", R Core Team 2018) was used to combine the resulting sequence table with sample-wise metadata and the count and taxonomy of each sequence by comparing them with the Green Genes Taxonomy database. A "phyloseq object" was made, which contained an average microbiome profile for each individual. This was done by combining metadata and adding up the counts of each bacterial OTU (operational taxonomic unit, comparable to species) from all samples from an individual with the merge_samples function in the 'phyloseq' package. Since many individuals were caught and sampled multiple times, we chose to pool together the results of all adult or sub-adult samples per individual, to get the final microbiome similarity index used in analyses. For example, if an individual was caught both as an adult and sub-adult, only adult samples were used. This was done because sub-adult and juvenile individuals often have a divergent microbiome compared to adults, and excluding these samples reduced variation related to age. The only reason we used juvenile or sub-adult samples was if the individual had only been sampled as a juvenile or sub-adult. Since diversity estimates are highly influenced by read depth, read counts were normalized by transposing them into relative abundances per sample. Outlier samples with exceptionally low read depths (n=300) and taxa known not to be gut bacteria (*Cyanobacteria, Xanthomonadales,* Mitochondria) were dropped from the data.

2.4. Statistical analyses

Making matrices

The pedigree was used to create a relatedness matrix using the function makeA from R package 'nadiv' (Wolak 2012). The values in the matrix range from 0 to 1, where unrelated individuals have a relatedness coefficient of 0, half-siblings have a relatedness coefficient of 0.25, mother-pup, father-pup, and full sibling pairs have a relatedness coefficient of 0.5 and individuals in relation to themselves have a relatedness coefficient of 1. I constructed two relatedness matrices based on the accuracy of relatedness estimates derived from COLONY; one where the probability of specific

pairs was at least 95 % and another where the probability of specific pairs was at least 80 % (COLONY calculates the likelihood of how accurate the estimates are, giving a percentage next to each pair). I used the more accurate matrix (95 %) in my final analyses.

The microbial data were turned into microbiome similarity matrices by my co-supervisor Aura Raulo in Oxford. After normalizing the data as relative abundances (the frequency of sequence counts of each OTU relative to the total count within a sample), sample compositions were described as pairwise dissimilarity indices. These indices describe the relative differences of microbial communities between a pair of individuals based on abundance (Bray–Curtis dissimilarity index) or presence-absence (Jaccard index) of bacteria. The Bray–Curtis dissimilarity index is an index used to calculate the weighted compositional dissimilarity of species between two sites (or samples). It is calculated in the following way:

$$BC_{\alpha\beta} = 1 - \frac{2C_{\alpha\beta}}{S_{\alpha}+S_{\beta}},$$

where $C_{\alpha\beta}$ is the sum of the lesser counts of each OTU present in both samples α and β, and S_{α} and S_{β} are the complete number of OTUs present in both samples (Legendre & Legendre 2012). The Jaccard index, on the other hand, is used for comparing the non-weighted similarity of sample sets. It measures the proportion of total OTUs found in two individuals that they share. It is calculated in the following way:

$$S_{ij} = \frac{p}{p+q+r},$$

where p is the number of OTUs found in both sets, q is the number of OTUs found only in one set and r is the number of OTUs found only in the other set (Borcard et al. 2018). For convenience, the dissimilarity/distance values were converted into similarity indices, to make them intuitively comparable to the relatedness matrix and to see if they were positively correlated with the relatedness data. This was done by simply subtracting all the matrix values from one (for example Bray_matrix← 1-(as.matrix(BrayCurtis)).

Gut microbial composition and relatedness

I performed all statistical analyses using R (R version 3.5.1, "Feather Spray", R Core Team 2018). A Mantel test was used to test correlations between the relatedness matrix and the microbiome similarity matrices and to get results on a quantitative scale (whether relatedness as a continuous variable was associated with microbiome similarity as a continuous variable). A Mantel test is a permutational test, that measures the correlation between two matrices typically containing

measures of distance or similarity. Statistical significance is tested by randomly permuting the rows and columns of one matrix multiple times and comparing the correlation coefficients to those observed for the original matrix. The p-value is calculated as the number of permutations that lead to a higher than the observed correlation coefficient. The matrix contains $\frac{n(n-1)}{2}$ amount of distances and Mantel r-values can fall within a range between -1 to 1. An r-value of -1 suggests a strong negative correlation, 0 suggests no relationship at all and 1 suggests a strong positive relationship. (Legendre & Legendre 2012).

Controlling for other factors

After using the Mantel test, the decision was made to split both the relatedness matrix and the microbiome similarity matrices (Bray-Curtis and Jaccard) between the populations, because the two data sets differed tremendously in microbiome composition. Since the differences are due to the samples coming from different sites and separate sequencing batches, biological (site) and technical (laboratory) effects cannot be distinguished from each other and therefore it was logical to separate them. To find out which factor affected microbiome similarity most, the new population-specific matrices along with the trapping data were transformed into tables of pairwise data, resulting in two tables all together (Example Table 3). The columns in the table were called “pair”, “relatedness”, “Bray”, “Jaccard”, “sex” and “age”. The pair column had the ID’s of two individuals (for example M919+M928), relatedness had their relatedness coefficient, Bray and Jaccard had the different microbiome similarity indices of the two individuals (ranging from 0 to 1) and sex and age either had “Same” if the individuals were the same sex or age, or “Different” if they were not.

Table 3. The beginning of one of the pairwise tables made for analysis.

Pair	Relatedness	Bray	Jaccard	Sex	Age
M642 + M662	0.25	0.56155253	0.39038793	Same	Same
M642 + M684	0	0.33240414	0.16277299	Same	Same
M642 + M699	0	0.48518406	0.22029242	Different	Same

A linear model was then fitted to each pairwise table, to find out which factor (sex, age or continuous relatedness) predicted pairwise microbiome similarity the most. Linear models depict a continuous response variable as a function of one or more predictor variables. It can be calculated in the following way:

$$y_i = \alpha + \beta x_i + \varepsilon_i$$

where y_i is the response variable (microbiome similarity value), x_i is a vector of the explanatory variables (sex, age, and relatedness), α (intercept) and β (slope) are the unknown parameters that we want to estimate and ε_i is the residual or deviation between the measured response variable and the prediction of the model. The linear model assumes that residuals are normally distributed (Legendre & Legendre 2012). The Anova test from R package 'car' was then used with parameter type = 3 to determine the importance of the factors (Fox & Weisberg 2011). The Anova test analyzes the amount of variance that is contributed to a sample by different factors. Type= 3 means each factor is looked at as if it were added to the model last, eliminating the importance of the order factors are added in the model (Legendre & Legendre 2012).

Because the effect of relatedness was weaker in Silwood, which also had more microhabitat variation and more microbiome variation, I decided to see if spatial variation (geographical location of host mouse territory) helped explain microbiome similarity as well, due environmental transmission of bacteria. The home range centroid of each mouse in both populations was calculated as the weighted mean of x- and y-coordinates of all locations an individual had been observed in during trapping. The resulting numbers were made into two population-wise matrices, which were added as columns to the population-wise data tables (Example table 3). The values in the column were distances (in meters) between the centroids of two individual's home ranges. The same linear model was fitted and executed again (for both the Bray-Curtis and Jaccard metrics), only this time spatial distance was added as a covariate as well. Finally, the Anova test from R package 'car' was used with parameter type = 3 to see if spatial distance could help explain variation between microbiome composition of individuals.

Does the type of relationship matter?

To test for differences in microbiome similarity between different types of relatives, I categorized pairs in the relatedness matrices into their own groups. The values in the relatedness matrix were changed to the 5 following relationship categories: Pairs with a relatedness coefficient of 0.5 were separated into "mother-pup", "father-pup" and "full sibling" pairs, pairs with a relatedness coefficient of 0.25 were called "half-siblings" and pairs with a relatedness coefficient of 0 were called "unrelated". The categorization was done based on the results from COLONY.

Since I was mostly interested in the effects and differences of close relatives (mother-pup, father-pup, and full siblings), the following columns were added to the data tables to indicate the type of relationship for each pair (Example Table 3): "mother-pup", "father-pup" and "fullsib". The binary values in these columns were set as either "yes" or "no", based on the relationship between

the pair of individuals (a mother and its pup had "yes" in the mother-pup column, "no" in the two other columns, etc.). Half siblings were excluded from the analysis, although they were visualized in figures 2, 6 and 7 for reference.

A linear model was fitted to the tables again, this time using sex, age, spatial distance, mother-pup, father-pup, and fullsib as explanatory factors for microbiome similarity. Four separate models were made and backwards pairwise elimination was carried out by removing one of the relatedness categories at a time. The base R ANOVA test was then used to compare each reduced model to the model with all factors, to see which factor affected the model fit most. To further see whether similarity to the mother was different from similarity to the father across populations, a subset of data containing only mother-pup and father-pup pairs from both populations was made. Another linear model was run with the same control factors as before (sex, age and spatial), only this time "relatedness" (mother-pup and father-pup) was used as well. The base R ANOVA test was used to analyze variance between mother-pup and father-pup pairs in the model.

Note: Since my data units are dyads, they are not independent of each other, which is not accounted for by my linear model framework. However, there were no strong clustering or outliers within the populations in the microbiome data (see Figure 3), so treating these dyadic metrics as independent observations should not bias my results. A more rigorous modeling framework that accounts for non-independence in dyadic data might provide more detailed insights into all relationships explored in the future. All statistical analyses were done by me, but I had help with some of the plotting codes from my co-supervisor Aura Raulo.

3. Results

3.1. General description of wood mouse population genetics and microbiome

Of the 138 mice genotyped, microbiota sequencing failed for ten individuals, which were consequently excluded from downstream analyses. The final dataset, therefore, included 58 mice from Wytham (28 females and 30 males) and 70 mice from Silwood (30 females and 40 males) with both genotypic and matching microbiota data.

The most common category of relatedness found in both populations in the data were half-sibling pairs (Figure 2). They composed 1.3 % of the data collected from Wytham and 1.1 % of the data collected from Silwood. The rest of the data collected from Wytham consisted of mother-pup pairs (0.5 %), full siblings (0.4 %), father-pup pairs (0.3 %) and more distant relatives/unrelated individuals (97.5 %). The rest of the data collected from Silwood consisted of mother-pup pairs

(0.7%), father-pup pairs (0.6 %), full siblings (0.5 %) and more distant relatives/unrelated individuals (97.1 %). Therefore, the individuals sampled in Silwood were more related to each other than the individuals sampled in Wytham.

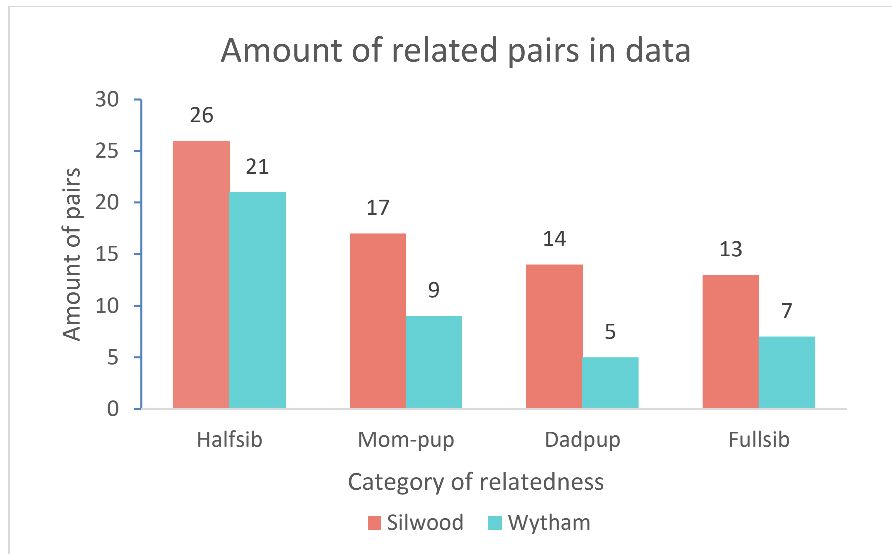

Figure 2. This graph shows the number of related pairs found in both populations in the relatedness matrix. Unrelated and more distantly related pairs have been excluded from the graph.

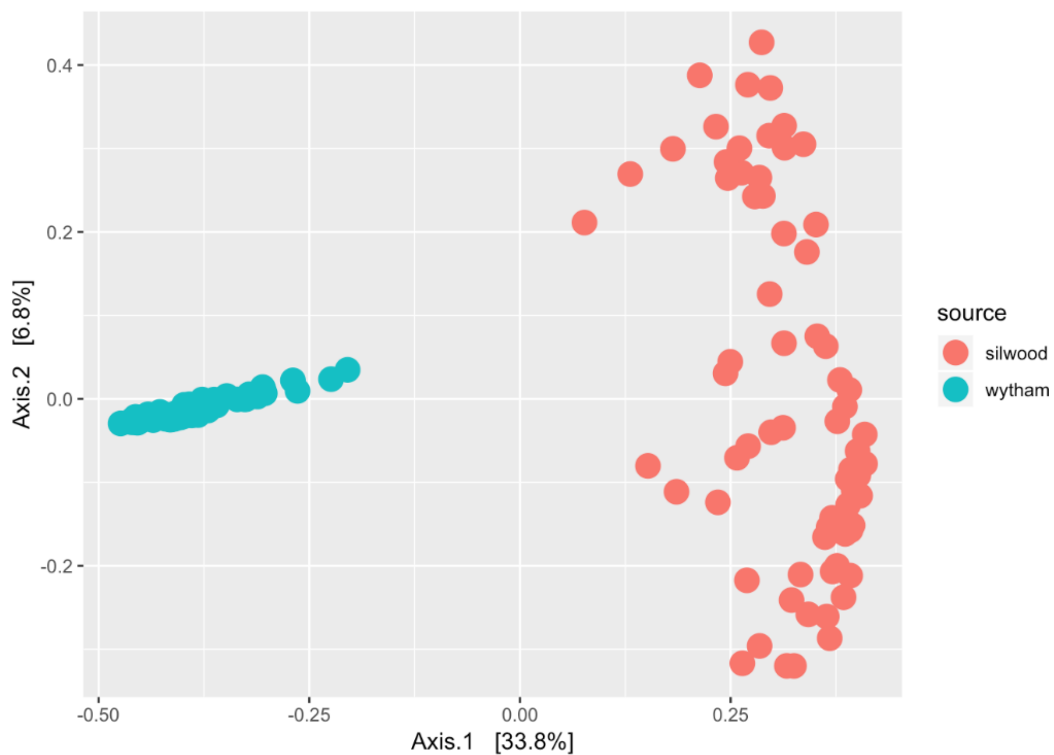

Figure 3. Principal coordination analysis (PCoA) of Bray-Curtis microbiome similarity within both populations.

The microbiome samples used for analysis were mostly taken from adult individuals (70.3% adult, 23.4 % sub-adult and 6.3 % juvenile). The microbiome data was visualized using principal coordinate analysis or PCoA (Figure 3). PCoA aims to find the axis along the multidimensional distribution that displays the most variation (Legendre & Legendre 2012). The microbiome data from my samples clustered clearly and unsurprisingly according to the source population. In Wytham, the microbiome composition of individuals seemed to be more similar within the population, than it was in Silwood. Wood mice guts, like the guts of other mammals, are mostly dominated by the phyla Firmicutes, Bacteroidetes and Proteobacteria, though assumed pathogens are also quite copious (Maurice et al. 2015). This could be seen in my data as well (Figure 4), where Firmicutes were the most abundant in both populations, though a bit more common in Silwood.

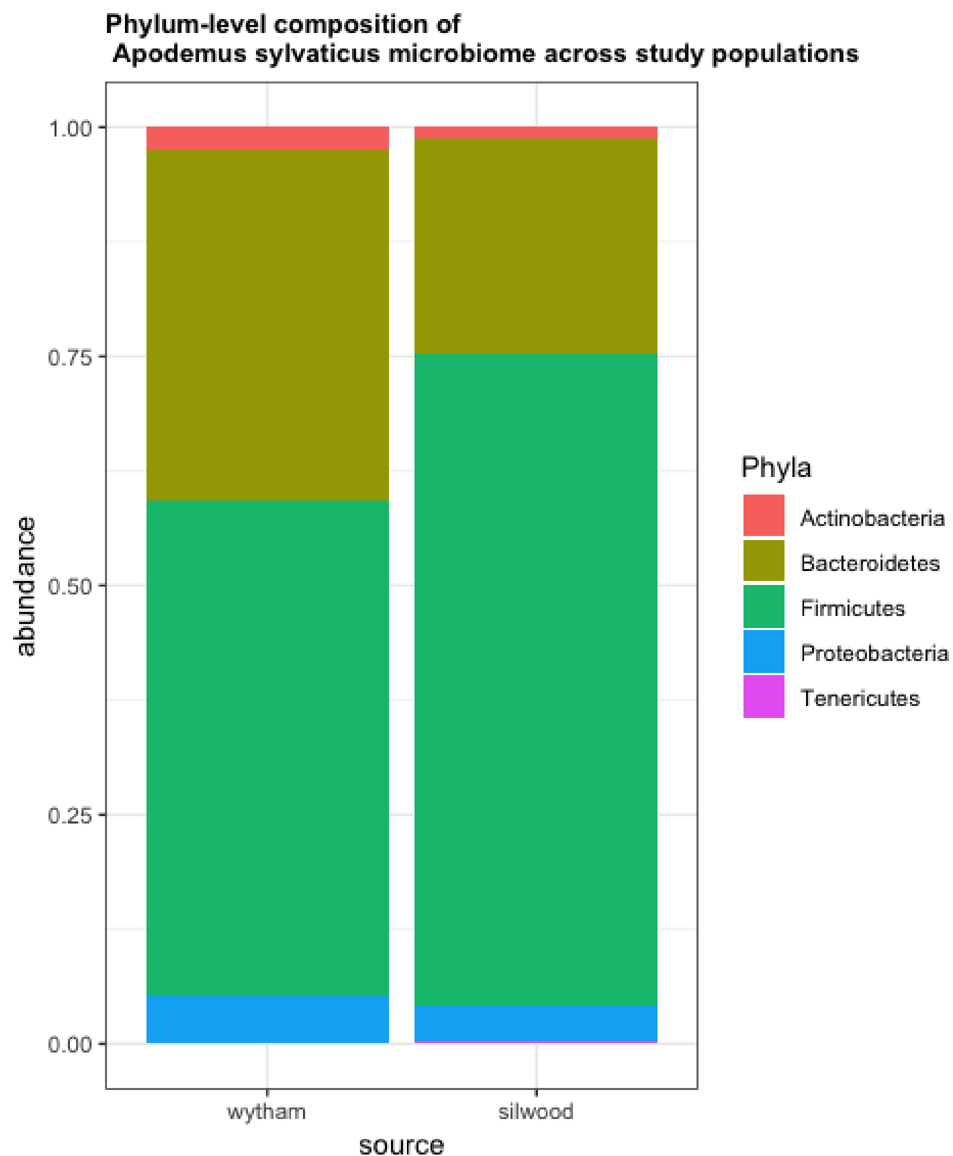

Figure 4. *The phylum-level composition of wood mouse microbiomes in both Silwood and Wytham.*

3.2. Gut microbial composition and relatedness

Gut microbial similarity was correlated with relatedness, but this effect depended on source population. Figure 5 illustrates the significant positive correlation between microbiome similarity and relatedness in the Wytham population and the non-significant and weak correlation in the Silwood population when using the Bray-Curtis microbiome similarity matrix. Specifically, relatedness predicted 12.9 % of microbiome similarity in Wytham (Mantel test $r = 0.1293$, $p < 0.01$), but had no significant effect in the Silwood population (Mantel test $r = -0.01862$, $p = 0.70363$). The trends were similar with the Jaccard microbiome similarity matrix as well, with relatedness predicting 12.9 % of microbiome similarity in Wytham (Mantel test $r = 0.1293$, $p < 0.01$) and no significant effect in Silwood (Mantel test $r = -0.03511$, $p = 0.87321$).

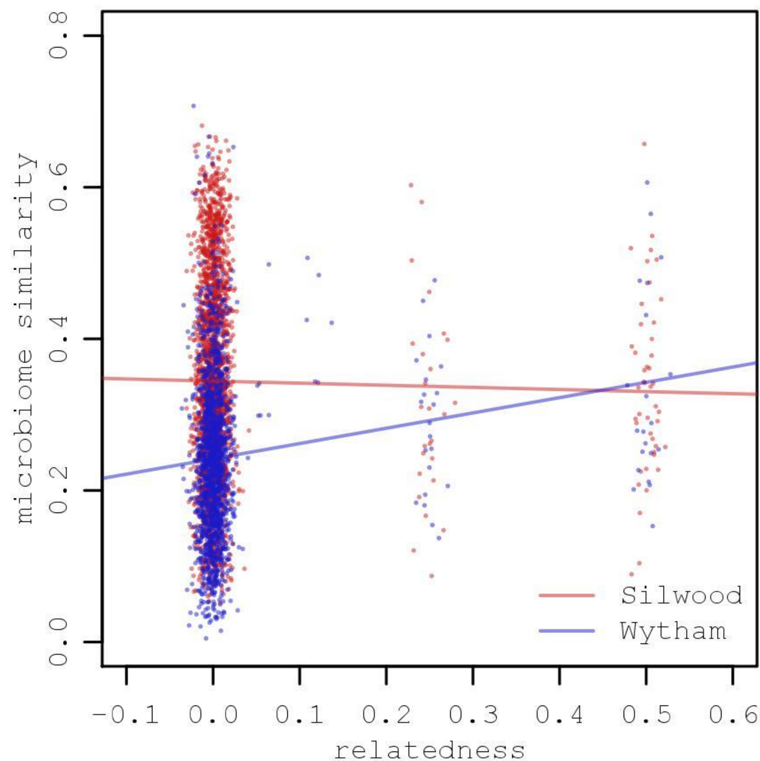

Figure 5. Microbiome similarity in relation to relatedness in both Silwood and Wytham populations. Microbiome data calculated using the Bray-Curtis index.

3.3. Effect of other covariates

According to the results from analysis done with both the Bray-Curtis and Jaccard data tables, microbiome similarity in the Wytham population was not influenced by the sex of individuals (Anova test $p = 0.9007614$ with Bray-Curtis, $p = 0.9688023$ with Jaccard, Table 4). However, samples from individuals with the same age category had more similar microbiomes than individuals that were a different age (Anova test $p < 0.01$ with both Bray-Curtis and Jaccard).

Relatedness also affected microbiome similarity significantly in Wytham when sex and age were controlled for, and the microbiomes of closely related individuals were more similar than those of unrelated ones (Anova test $p < 0.01$ with both Bray-Curtis and Jaccard).

Table 4. Output for the Anova test (R package 'car') for the Wytham population. Done above with Bray-Curtis microbiome similarity and below with Jaccard microbiome similarity.

Anova(lm(microbiome_similarity~sex+age+relatedness, type = 3)

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	22.593782	1	2065.9770	< 2.2e-16	***
sex	0.0001701	1	0.0155548	0.9007614	
age	0.5067751	1	46.339553	< 2.2e-12	***
relatedness	0.2823144	1	25.814847	0.0000004	***
Residuals	18.033669	1649			

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	7.5380160	1	1476.2648	< 2.2e-16	***
sex	0.0000078	1	0.0015301	0.9688023	
age	0.2432306	1	47.634915	< 2.2e-12	***
relatedness	0.1387470	1	27.172573	0.0000002	***
Residuals	8.4200264	1649			

The results from the Silwood population were a bit different (Table 5). Analysis with both the Bray-Curtis and Jaccard data table showed that sex did, in fact, have an effect on microbiome similarity when individuals were of the same sex (Anova test $p < 0.01$). Individuals of the same age also had significantly more similar microbiome composition than individuals that differed in age (Anova test $p < 0.01$ with both Bray-Curtis and Jaccard). In contrast, relatedness did not affect microbiome similarity significantly (Anova test $p = 0.6949711$ with Bray-Curtis, $p = 0.6136505$ with Jaccard), even when sex and age were controlled for.

Table 5. Output for the Anova test (R package 'car') for the Silwood population. Done above with Bray-Curtis microbiome similarity and below with Jaccard microbiome similarity.

Anova(lm(microbiome_similarity~sex+age+relatedness), type = 3)

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	74.678229	1	4257.084	< 2.2e-16	***
sex	0.1298066	1	7.3997177	0.0065702	**
age	1.6646425	1	94.894101	< 2.2e-16	***
relatedness	0.0026979	1	0.1537933	0.6949711	
Residuals	42.294022	2411			

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	27.989104	1	2803.5584	< 2.2e-16	***
sex	0.0859624	1	8.6105123	0.0033739	**
age	0.9644995	1	96.610121	< 2.1e-08	***
relatedness	0.0025454	1	0.2549595	0.6136505	
Residuals	24.070028	2411			

The additional tests done with the spatial data in both Wytham and Silwood (see Supplementary Tables 1 and 2), resulted in a significant effect for home range with both Bray-Curtis (Anova test $p < 0.01$ both populations) and Jaccard (Anova test $p < 0.01$ both populations), meaning the closer the individual's territories were to each other, the more similar their microbiome composition was.

3.4. Does the type of relationship matter?

Microbiome similarity was structured the same way in both populations (Figures 6 and 7): unrelated pairs varied fairly in microbiome similarity, but full siblings and mother-pup pairs seemed to share the most similar microbiomes in the data set. In contrast to this, fathers differed from their offspring even more than unrelated pairs did, implying that father-pup pairs had the least similar microbiomes in the data set. In both populations, offspring microbiome composition resembled the mother more than the father (ANOVA test $p < 0.05$ for both Bray-Curtis and Jaccard).

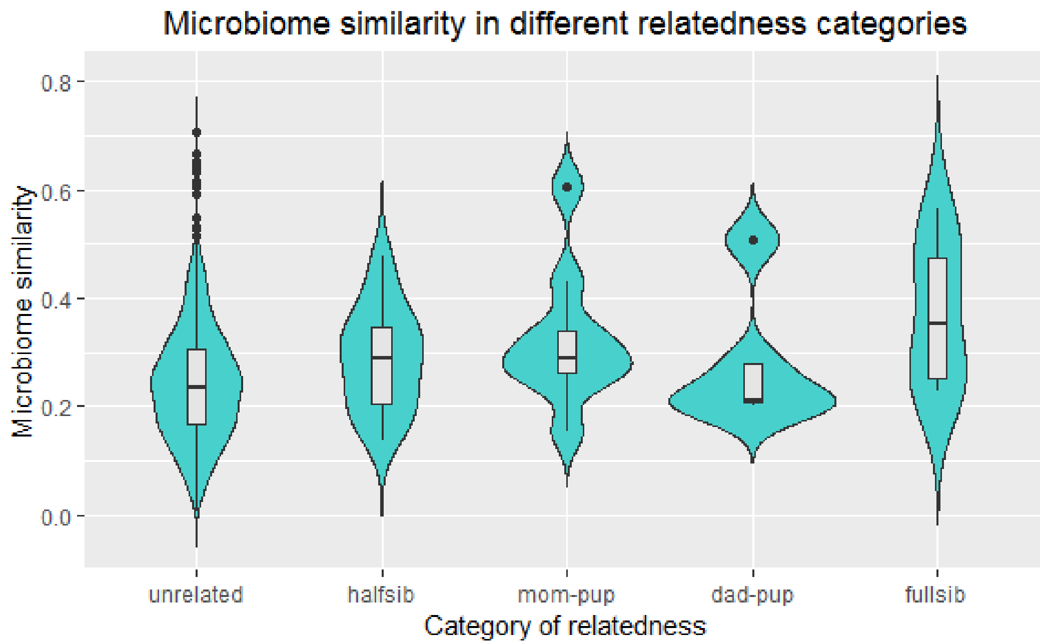

Figure 6. Bray-Curtis microbiome similarity in different relatedness categories in Wytham.

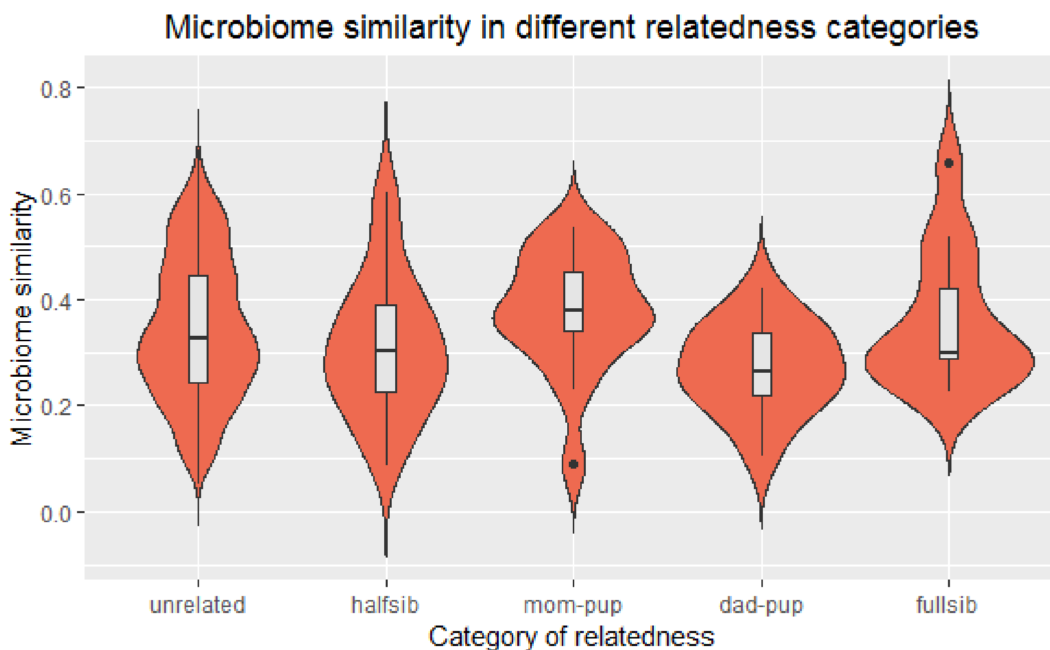

Figure 7. Bray-Curtis microbiome similarity in different relatedness categories in Silwood.

Results from Wytham (Table 6 and 7) show that both full siblings (ANOVA test $p < 0.01$ with both Bray-Curtis and Jaccard) and mother-pup pairs (ANOVA test $p < 0.05$ with both Bray-Curtis and Jaccard) had a significantly more similar microbiome than the rest of the pairs in the data. Similarity to the father's microbiome was lower but not significantly different from similarity to a unrelated pair's microbiome (ANOVA test $p = 0.4628$ with Bray-Curtis, ANOVA test $p = 0.4467$ with Jaccard).

Table 6. Output for the ANOVA test for the Wytham population done with the Bray-Curtis microbiome data.

Model 1: Bray ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Bray ~ sex + age + spatial + dadpup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	1646	17.800					
2	1646	17.848	1	-0.047673	4.4085	0.03591	*

Model 1: Bray ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Bray ~ sex + age + spatial + mompup + dadpup

	Res.Df	RSS Df	Df	Sum of Sq	F	Pr(>F)	
1	1646	17.800					
2	1647	17.912	1	-0.11197	10.354	0.001317	**

Model 1: Bray ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Bray ~ sex + age + spatial + mompup + fullsib

	Res.Df	RSS Df	Df	Sum of Sq	F	Pr(>F)
1	1646	17.800				
2	1647	17.806	1	-0.0058327	0.5394	0.4628

Table 7. Output for the ANOVA test for the Wytham population done with the Jaccard microbiome data.

Model 1: Jaccard ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Jaccard ~ sex + age + spatial + dadpup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	1646	8.3142					
2	1647	8.3389	1	-0.024771	4.9041	0.02693	*

Model 1: Jaccard ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Jaccard ~ sex + age + spatial + mompup + dadpup

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	1646	8.3142					
2	1647	8.3729	1	-0.058737	11.629	0.0006652	***

Model 1: Jaccard ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Jaccard ~ sex + age + spatial + mompup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)
1	1646	8.3142				
2	1647	8.3171	1	-0.0029259	0.5792	0.4467

In Silwood, microbiome similarity between siblings or mother-pup pairs was not significantly different from the mean similarity in the data (ANOVA test: sibling-pairs p = 0.6138, mother-pup pairs p = 0.1982) when using the Bray-Curtis data (Table 8). Father-pup pairs, on the other hand, had a nearly significant negative association (ANOVA test p = 0.05121), meaning that father-pup pairs had less similar microbiome than other pairs in the data. Similarly, the Jaccard data (Table 9) showed that microbiome similarity was significantly different between father-pup pairs

(ANOVA test $p < 0.05$), while mother-pup (ANOVA test $p = 0.2481$) and fullsib pairs (ANOVA test $p = 0.6306$) did not have a significant effect. The positive correlation between mother-pup and full sibling microbiome similarity and the negative correlation between father-pup microbiome similarity can be seen in both populations (Figures 6 and 7), although the effects are not significant in both populations.

Table 8. Output for the ANOVA test for the Silwood population done with the Bray-Curtis microbiome data.

Model 1: Bray ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Bray ~ sex + age + spatial + dadpup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	2408	42.580					
2	2409	42.609	1	-0.029288	1.6563	0.1982	

Model 1: Bray ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Bray ~ sex + age + spatial + mompup + dadpup

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	2408	42.580					
2	2409	42.584	1	-0.0045049	0.2548	0.6138	

Model 1: Bray ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Bray ~ sex + age + spatial + mompup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	2408	42.580					
2	2409	42.647	1	-0.067287	3.8053	0.05121	.

Table 9. Output for the ANOVA test for the Silwood population done with the Jaccard microbiome data.

Model 1: Jaccard ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Jaccard ~ sex + age + spatial + dadpup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	2408	24.226					
2	2409	24.239	1	-0.013427	1.3346	0.2481	

Model 1: Jaccard ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Jaccard ~ sex + age + spatial + mompup + dadpup

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	2408	24.226					
2	2409	24.228	1	-0.0023271	0.2313	0.6306	

Model 1: Jaccard ~ sex + age + spatial + mompup + dadpup + fullsib
Model 2: Jaccard ~ sex + age + spatial + mompup + fullsib

	Res.Df	RSS	Df	Sum of Sq	F	Pr(>F)	
1	2408	24.226					
2	2409	24.267	1	-0.040857	4.0611	0.04399	*

4. Discussion

4.1. Differences between the populations

There were significant differences between the Wytham and the Silwood population, which may be explained by multiple factors. Firstly, the individuals sampled in Silwood were more related to each other than in Wytham (Figure 2). In Silwood 2.9 % of the data consisted of related individuals, while in Wytham only 2.5 % of the individuals were related. This could mean Silwood had a smaller population size with less immigration or it could mean more related individuals were captured by chance, though this seems unlikely. Another difference between the populations was that in Silwood, gut microbiome composition seemed to vary more within the population than in Wytham (Figure 3). This transverse variation may be caused because samples for both populations were extracted by different people and sequenced in separate batches, which may affect the outcome of sequencing and produce slightly different results. Furthermore, Silwood had a larger trapping grid with a more heterogeneous habitat than Wytham (See Supplementary Figures 1 and 2). Although both trapping grids were 2.4 hectares large in the end, roughly half of the Wytham samples were captured when the grid was 1.4 hectares smaller. In addition, Silwood consists of several microclimates (bamboo, rhododendron, etc.) that likely harbor different soil bacterial taxa. Since diet and the environment in general play an important role in microbiome composition (Benson et al. 2010; Maurice et al. 2015), it is fair to assume these factors increase variation in microbiome composition within the Silwood population. This can be seen in the PCoA visualization (Figure 3), where the x-axis could represent environmental effects on the microbiome. This notion is also corroborated by models showing that spatial distance among mouse home range centroids explained a significant proportion of individual gut microbiome variation.

Bray-Curtis vs. Jaccard

Classifying microbiome similarity using Bray-Curtis or Jaccard matrices gave slightly different results. Since the Bray-Curtis index accounts for relative abundance and the Jaccard index accounts for presence-absence, this was not unexpected. If the model with Jaccard index yielded significant results for a certain variable, pairs shared many bacterial taxa, whereas the model with Bray-Curtis yielding significant results implied that pairs shared many bacterial taxa in similar quantities. Therefore, if a result was significant for Jaccard index but not Bray-Curtis, this would suggest the effect was likely driven by rare bacterial taxa. For example, in Silwood father-pup pairs had a significantly different microbiome with the Jaccard index, but the effect was only nearly significant

with the Bray-Curtis index. In general, the Jaccard index was more likely to result in a significant outcome but yielded a less specific result.

4.2. Gut microbial composition and relatedness

Overall, related individuals had more similar gut microbiomes than unrelated individuals, though on a continuous scale this effect was only significant in Wytham. This could be due to both genetic effects on the microbiome and/or transmission among close relatives, especially from mother to pups. The mammalian gut is a product of gene expression and it can be seen as a secluded ecosystem, with one way in and one way out. For a microscopic organism, different parts of the gut are like patches in a macroecological ecosystem and variation in factors such as mucus secretion, acidity or the epithelial layer affect how the microbe communicates with and adapts to its surroundings (Chassaing et al. 2014). All these factors are more or less affected by host genotype. For example, immune genes of the host regulate composition and temporal variations in the microbiome, making host genotype an important factor affecting microbiome composition and stability (Levy et al. 2015). Therefore, relatives are likely to share a more similar microbiome, since host genotype selectively shapes the microbial community directly through controlling immunogenotype, much like abiotic factors control a macroecological ecosystem (Costello et al. 2012).

In a semi-social species maternal care, close sibling interaction, and relatives sharing similar microbiota can also be a product of transmission: In line with the mass-effect perspective in metacommunity theory, immigration and emigration of bacteria connect the metapopulations inside the gut, helping maintain diversity within the microbial community. This also explains variation between related individuals that interact with each other: For example, baboons are known to groom their close relatives more and mutual grooming is associated with their microbiome similarity (Tung et al. 2015). Furthermore, genotype alone is by no means the strongest predictor of microbiome composition: Even monozygotic twins differ in gut microbial communities (Goodrich et al. 2014). Transmission is an important shaper of the microbiome because it is the primary source of microbial diversity, which is important to host immune system. A diverse microbiome is also the most stable (Lozupone et al. 2012) and even after a disturbance such as disease or ingestion of antibiotics, patches that remain untouched and healthy can act as sources of bacteria to the rest of the gut, helping revive and redistribute mutualistic bacteria (Leibold et al. 2004).

Unrelated individuals with different alleles can be viewed as separate patches on a larger scale, that differ in microbiome composition because of patch quality and limited dispersal,

consistent with the species-sorting perspective (Leibold et al. 2004). The more an individual interacts with its peers, the more similar their microbiomes become (Tung et al. 2015; Raulo et al., 2018), yet all of this happens within the boundaries set by the host's genotype. A host suffering from a genetically affected disease such as Crohn's disease may be exposed to mutualistic bacteria, but the bacteria cannot colonize the gut if the environment is hostile towards it (Tysk et al. 1988). In addition, host individuals with beneficial genes that induce a healthy microbiome (e.g. stable) are at an advantage when it comes to survival since they are more likely to be healthy and pass on their genes to their offspring (Zilber-Rosenberg & Rosenberg 2008).

In Silwood, there was no significant correlation between microbiome similarity and relatedness. The microhabitats that form the trapping area in Silwood can be seen as patches of different quality, that have various abiotic (pH, salinity, texture, etc.) and biotic (plant and animal species) factors affecting what bacteria can thrive in each patch. The mice that live and wander through these patches are colonized by the bacteria they encounter on the way. The constant immigration of bacteria into the gut through food and contact with soil and other organisms increases variation between individuals within the population, even if their territories overlap (Maurice et al. 2015; reviewed by Fierer 2017). Since wood mice are territorial and their home ranges are exceptionally stable and small (Godsall 2015), it is no wonder that fine scale microhabitat variation in the Silwood trapping site (see supplementary figure 2) was associated with variation in microbiome composition. This is quite in tune with the species-sorting perspective because the microbiome is not only affected by the environmental quality of the patch but the dispersal of mice as well.

As mentioned before, the two populations varied immensely in comparison to each other. While much of this variation is likely due to sequencing batch effect, some is likely accountable for true area differences, which yet again corroborates the results of earlier studies on environmental effects on the microbiome (for example Benson et al. 2010). This is also supported by the metacommunity theory since the two habitats are isolated macroecological patches, that harbor different bacteria because of the different biotic and abiotic factors found in the separate areas. Adaptation is favored when dispersal rates in a patch are low (Costello et al. 2012) and since members of the two populations do not interact, the mice and their microbiomes have adapted to their own habitats accordingly. This is interesting since within this one result we can find three different levels of ecological communities that all affect the outcome. These levels are the gut of the host that is affected by host genotype, the microhabitats that are affected by abiotic factors and

stochastic dispersal of species and the macro habitats or trapping areas, that are affected by abiotic factors but are also a sum of all the microhabitats inside.

4.3. Drivers of gut microbial composition

Wytham

After area and relatedness (the effect of which was amplified when control factors were added to analysis), the main factors explaining gut microbiome similarity of wood mice in Wytham were age and home range. Adult individuals had more similar microbiomes than adult and juvenile/sub-adult pairs, which makes sense since the microbiome is known to develop and stabilize over time (Lozupone et al. 2012). A climax community is diverse and stable and is not immediately destroyed by changes in the ecosystem (Clements 1916). Adult individuals can have a core microbiome, that consists of the same bacterial taxa that establish in the gut over time (Tap et al. 2009), and thus the effect of early determinants of microbiome composition can be expected to be seen at least in a presence/absence scale (Jaccard index) in adult individuals. In Wytham, the trend was so strong that it could be seen on the abundance scale as well (Bray-Curtis index). The correlation between mouse home range location and microbiome composition is also a logical driver of microbiome similarity since the closer individuals live to each other, the more they are exposed to the same environmentally transmitted soil bacteria (Reviewed by Spor et al. 2011; reviewed by Fierer 2017).

In contrast to previous studies (for example Bolnick et al. 2014), sex did not affect microbiome similarity in Wytham. Not much is known about the effect of sex on gut microbiome composition, other than it is most likely regulated by hormonal changes especially after puberty (Martin et al. 2016). Similarly, pregnant females have been found to differ from non-pregnant females in gut microbiome composition in both red-bellied lemurs and humans (Koren et al. 2012; Raulo 2015). A few pregnant individuals were caught during trapping and since individual microbiome samples were pooled together, samples from pregnant mice could have shifted the balance of species abundance among female individuals and affected the results. Another option is, of course, that wood mice do not have a sex-specific microbiome: It is possible that hormonal effects on the microbiome in wood mice are non-existent, or weak under the effect of all the other explanatory factors.

Silwood

In addition to area, age and home range, that explained microbiome similarity in both populations, microbiome similarity was also affected by sex in Silwood. Since males and females have different sex chromosomes, they differ partly in genetic makeup and hormonal secretion, which affects gut

microbial composition among other things (for example Gomez at al. 2015). Male and female guts can, therefore, be seen as patches with different genetically encoded immunological and hormonal factors affecting the microbial community. Sex differences have been found to result in sex-specific microbial clades, that also affect the fitness of the individual (Gomez et al. 2015). For example, increased testosterone levels in male mice have been found to amplify colonization of the gut by segmented filamentous bacteria, which prevent type 1 diabetes (Yurkovetskiy et al. 2013). It appears that in Silwood individuals of the same sex had more of these sex-specific taxa in common, making sex a significant driver of microbiome similarity, despite the non-existent effect in Wytham.

Although Silwood consists of various microhabitats and home range did have a significant effect on microbiome similarity, controlling for spatial variation did not make the effect of continuous relatedness significant. This might be due to mother-pup pairs and father-pup pairs pulling the effect in opposite directions (mother-pup pairs had very similar microbiomes and father-pup pairs had the most differing microbiomes). If this is the case, there might be an underlying kinship effect on microbiome similarity in both populations, but because father-pup pairs are not more similar than unrelated pairs, this effect could be driven by maternal transmission. Whatever the case may be, these differences in microbiome similarity can be explained through metacommunity theory.

4.4. Does the type of relationship matter?

Mother knows best

In both populations, offspring shared a more similar microbiome with their mothers than fathers. Even in Silwood, where no significant difference was found between mother-pup and unrelated pairs, the positive effect of mothers on microbiome similarity was still stronger than that of fathers. This corroborates my hypothesis that maternal transmission of bacteria at birth has a significant effect on the microbiome composition of an individual. The full effect of the mother on offspring microbiome in wood mice consists of maternal transmission, genetics, and contact with the offspring (Wolf & Wade 2009; Benson et al. 2010), while the father only acts as a donor of genes (Flowerdew & Tattersall 2008). Coupled with the lack of evidence for pure genetic effects on microbiome composition, the higher similarity between mother-pup pairs can be explained by maternal transmission and postnatal physical contact.

Maternal transmission is comparable to “priority effect” or the first colonization of an ecosystem after it has formed. When maternally transmitted bacteria colonize the gut, they ensure that the microbial community within the gut becomes diverse and healthy (Bennet & Nord 1987). If

for some reason the gut is first colonized by other bacteria instead, the beneficial symbionts may not be able to find space to settle, resulting in a completely different microbiome composition. This is not unlike the succession of plants after a forest fire; random chance may affect what species arrive at the new habitat first and species that normally wouldn't thrive in the conditions may be able to establish themselves, because of the lack of competition (Clements 1916). Since wood mice only give birth vaginally (and not through c-section like humans can), the offspring always receives its first bacteria from the mother's birth canal, giving maternally transmitted bacteria "priority effect". The effect lasts well into adulthood, which is supported by my results: mother-pup pairs shared similar microbiome composition, despite 70.3 % of sampled individuals being adults.

Maternally transmitted bacteria set the course for the consequent microbiome development. While pregnant and in preparation for birth, the composition of a human mother's vaginal microbiome becomes less diverse and dominated by species of *Lactobacillus*, *Bacteroidales* and *Bifidobacterium* (Dominguez-Bello 2010; Aagaard et al. 2012), which help digest milk and prevent acute diarrhea (Liepke et al. 2002). Certain proteins produced in human milk have been found to have no other function than to feed strains of *Bifidobacterium* (Sela et al. 2008), proving again that these bacteria are meant to colonize the gut in the early stages of an individual's life. This evidence shows there is a long evolutionary history between the host and its symbionts and that even the microbiome of the mother is primed to maximize the fitness of the offspring.

Fathers and siblings

The fact that fathers had a divergent microbiome composition compared to their offspring may appear counterintuitive at first, especially in Silwood, where father-pup pairs had significantly contrasting microbiome composition compared to all other pairs. Not only did father-pup pairs have less similar microbiomes than other related pairs, but they differed more than unrelated pairs in general. This could simply be due to fathers not having contact with their offspring or the age gap between father and offspring since age was found to affect microbiome similarity significantly in both populations. Alternatively, mothers might intentionally choose to mate with males that have a dissimilar immunogenotype (disassortative mating). Mating non-randomly based on alleles underlying immunity has been found in both mammals (Huchard et al. 2013) and birds (Løvlie et al. 2013) and since this strategy could potentially enhance an offspring's parasite resistance, it is an interesting theory to consider.

Unlike father-pup pairs, full siblings are the same age, which could help explain why full siblings shared the most similar microbiome composition in both Wytham and Silwood (Figure 6 & 7). Not only do full siblings share age, half of their genes and the same maternally transmitted

bacteria, but they sometimes even overlap in territory (Godsall 2015), making it logical that they had the most similar microbiome composition out of the dataset.

4.5. Future directions

There is still much to learn about how host genotype and maternal transmission affect the gut microbiome. Future studies should focus on what microbes reside in the gut and try to differentiate maternally transmitted taxa from taxa varying within an individual lifetime (such as environmentally or socially transmitted taxa). One way of doing this is to use the Dufrene-Legendre indicator index, which is a simple method that identifies indicator species and species assemblages that characterize sites. The indicator combines the relative abundance of the taxa with its relative frequency of occurrence in the different sites (Dufrene & Legendre 1997). This can help identify which genera of bacteria are shared between mother-pup pairs vs. socially interacting pairs (social transmission, e.g. among siblings) or pairs of "neighbors" sharing the same microhabitat territory (environmental transmission), making it possible to distinguish the taxa that are the most affected by early-life vs. later transmission. Similarly, if more rigorous analyses reveal that a subset of microbiome variation is indeed affected by host genotype, similar indicator analyses can be used to distinguish the taxa whose abundance in the gut depends primarily on transmission vs. selective filtering processes induced by host genotype. For example, to distinguish the genetic effect from the maternal effect on the microbiome, one could identify bacterial taxa shared by all parent-offspring pairs minus taxa shared by only mother-pup pairs. The bacteria that are shared between mother-pup pairs but not father pup pairs are most likely to be maternally transmitted ones.

In addition, comparisons between juvenile and adult microbiomes should be conducted to further understand the relationship between maternally transmitted bacteria and the adult microbiome. This could be done by constructing a similarity matrix containing only juvenile microbiome data and constructing another one using adult microbiome data from the same individuals. Similar analyses to mine could be done to compare the results and to see whether the effect of maternal transmission or genotype is more pronounced in early life. One hypothesis could be that the effects of maternal transmission are more visible in juvenile individuals, but the effects of host genotype persist throughout a lifetime. Alternatively, another hypothesis could be that genetic or paternal effect is more visible in juvenile individuals and is lost over time in the "noise" of a more versatile adult microbiome.

5. Conclusions

This thesis solidified in the wild what many studies have concluded in captivity before: the microbiomes of wood mice seem to be affected by maternal transmission, but surprisingly, host genotype does not have an effect clearly independent of this. The origin of variation in a healthy microbiome composition has been a long-debated subject since it is a crucial part of host immunity and metabolism. Based on my results, transmission of bacteria during and shortly after birth is a key factor shaping microbiome composition, enhancing genetic effect and making the mothers influence stronger, just like predicted. In fact, maternal transmission may potentially account for the entire genetic influence found in my analysis, since unlike all other relatives, fathers differed in microbiome composition more than unrelated individuals in general.

Much like a secluded island, microbiome composition seems to be shaped by what species stochastically make it there first, instead of the genetic factors controlling the gut. Thus, priority effect seems to be more descriptive of microbiome assembly in wood mice than selective filtering by the host. There are several parallel examples from macroecological community assembly functioning the same way: For example, New Zealand did not use to have terrestrial mammals due to it breaking away from Gondwana and drifting off into isolation 85 million years ago (Parkes & Murphy 2003). This was not because it was an unsuitable environment for animals, plenty of birds and reptiles thrived there. It was only because of dispersal limitations that terrestrial mammals had to wait for humans to introduce them to the islands thousands of years later. Dispersal limitations seem to be the main driver in defining microbiome composition in my thesis, which raises the question, is this the case in previous studies as well? How much of the reported "genetic" effect on the microbiome is actually maternal or sibling-transmission effect?

Evolutionary biology has benefitted tremendously from microbiome research and the way it innovatively redefines individuality. The "holobiont" concept has united worlds both visible and hidden, and it has helped us gain a more comprehensive understanding of how individuals and ecosystems function. Using theories that were formerly thought to only apply to macro-scale communities has proven to be helpful in explaining the dynamics between a host and its symbiont community. Understanding this bi-directional relationship is vital when considering topics such as adaptation and evolution in a rapidly changing world. The environmental challenges that ensue us affect all organisms, thus biodiversity from all levels of life must be considered while trying to find solutions. Therefore, combining ecology, evolutionary biology, and microbiome studies seem like a good avenue for breaking down the barriers keeping us from understanding ecosystems as a whole and finding new revolutionary ways to resolve human-induced issues.

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8. Supplementary data

Supplementary Table 1. Output for the Anova test (R package 'car') for spatial variation in the Wytham population. Done above with Bray-Curtis microbiome similarity and below with Jaccard microbiome similarity.

Anova(lm(microbiome_similarity~sex+age+relatedness+homerange), type = 3)

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	13.290921	1	1235.7416	< 2.2e-16	***
sex	0.0003771	1	0.0350619	0.8514891	
age	0.5582823	1	51.907065	< 2.2e-12	***
relatedness	0.2391429	1	22.234641	0.0000026	***
homerange	0.3087357	1	28.705125	0.0000001	***
Residuals	17.724933	1648			

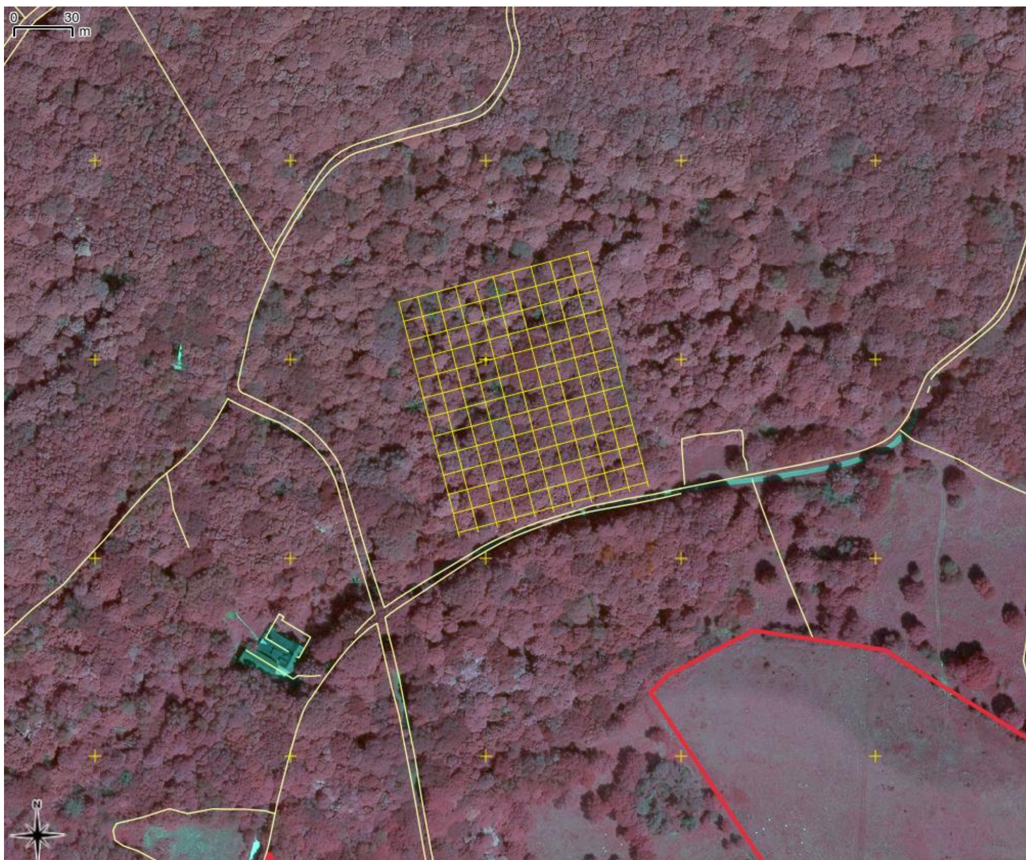
	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	4.5606164	1	906.7048	< 2.2e-16	***
sex	0.0000018	1	0.0003655	0.9847497	
age	0.2663536	1	52.954256	< 2.2e-12	***
relatedness	0.1189405	1	23.646785	0.0000013	***
homerange	0.1307843	1	26.001468	0.0000004	***
Residuals	8.2892421	1648			

Supplementary Table 2. Output for the Anova test (R package 'car') for spatial variation in the Silwood population. Done above with Bray-Curtis microbiome similarity and below with Jaccard microbiome similarity.

Anova(lm(microbiome_similarity~sex+age+relatedness+homerange), type = 3)

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	35.889765	1	2053.5340	< 2.2e-16	***
sex	0.1363007	1	7.798831	0.0052693	***
age	1.6377812	1	93.710269	< 2.2e-12	***
relatedness	0.0061479	1	0.3517685	0.5531694	
homerange	0.1742749	1	9.9716266	0.0016094	***
Residuals	42.119747	2410			

	Sum Sq	Df	F value	Pr(>F)	
(Intercept)	13.897835	1	1398.0572	< 2.2e-16	***
sex	0.0902102	1	9.0747219	0.0026186	***
age	0.9480958	1	95.374004	< 2.2e-12	***
relatedness	0.0051429	1	0.5173487	0.4720446	
homerange	0.1126530	1	11.332368	0.0007736	***
Residuals	23.957375	2410			



Supplementary Figure 1: Satellite picture of original Wytham trapping site (1 ha in size), consisting of open woodland with bramble bush patches.

Supplementary Figure 2: Map of the Silwood trapping site (2.4 ha in size), consisting of open woodland, patches of bamboo, rhododendron and other microhabitats.