Microbiota and species conservation: Drivers of gut microbial communities variation in the context of captive breeding programs

by

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Summary

Biology is undergoing a paradigm shift, where individual phenotypes are seen as a result of complex interactions resulting from the combined expression of the host and associated microbial genomes, leading to the popularization of the holobiont concept. The vast communities of microorganisms such as bacteria found in the mammalian gut have been shown to play vital roles in the growth and maintenance of healthy multicellular organisms, although the complex relationships between host and their associated microbes remain poorly understood. It is especially striking in endangered species, that face extreme conservation challenges and are less studied than livestock, pets or even humans.

Conservation breeding programs, involving periods of captivity and constrains in the mating system, can promote shifts in gut microbial communities in threatened species. Captivity especially implies veterinary care and prophylaxis, limited contact with other species and/or conspecifics, as well as diet and substrate change compared to natural environments. The use of drugs such as antibiotics and changes in diet are known to affect gut microbial communities in mammals. However, the shift from wild to captive environments can impact gut microbes differently according to each species, depending on their ecology and natural history. Studying those shifts is critical to understand at which depth conservation breeding programs impact the entire holobiont, both host and microbes, in order to promote each individual's reproduction and survival, and therefore the species' future.

In this PhD study, I investigated two broad classes of drivers of gut microbial communities' variation in threatened species under conservation breeding programs. Firstly, heritable drivers are potentially not reversible, or if so, over multiple host generations and therefore operate at long-term scales. Second, immediate drivers could induce variation in microbial community composition in a reversible manner and at the individual level of the host. I hypothesized that host genotype and birth location are heritable drivers, and host diet, biology (such as hibernation) and environment (captivity and geography) are short-term immediate drivers.

The taxonomic diversity and abundance of bacterial communities of the critically endangered Vancouver Island marmot's gut in the wild compared to *in* and *ex situ* facilities were investigated. Gut microbial diversity was higher in marmots held in *ex situ* facilities, outside of their habitat range, compared to captive marmots held within their habitat range, and in the wild and differences in composition were also observed. Microbial biomarkers and metagenome predictions indicated that those variations could be linked to diet change in raw foods between *in situ* and *ex situ* facilities.

I also conducted a longitudinal analysis in the pre- and post-hibernation periods and investigated the differences among marmots held at the *in situ* and *ex situ* facilities. Wild-born animals differed in their gut microbial communities compared to other marmots. In the pre-hibernation period, animals kept in the *ex situ* facility seem to have increased abundance in taxa that are metabolically versatile, compared to more abundant known mucin-degraders for *in situ* marmots. For the post-hibernation period, *ex situ* marmots tended to have more taxa associated with fiber degradation than *in situ* conspecifics. Knowing that *in situ* marmots have a gut microbiota similar to wild conspecific, these results confirm the interest to transfer animals held at the zoo at the *in situ* facility before relocation.

The phylosymbiosis theory was tested: I examined if host phylogenetic relatedness correlates with gut microbial communities' similarities between populations. The native European mink and the invasive American mink in Western Europe were used as models, and their gut microbiota was studied according to their relatedness and environment (captive or free-ranging). Our results showed differences between free-ranging and captive individuals, with more extreme changes in American mink compared to European mink, overshadowing species identity. However, feral American mink from a long-established population exhibited gut bacterial composition closer to the free-ranging native species compared to more recently established feral populations. This result could be explained by dietary shifts in the area sampled based on prey availability through different landscape, rather than greater genetic differentiation.

The impacts of host genotype (in MHC and neutral markers) on gut microbiota were investigated. I compared two distinct captive populations of the critically endangered European mink with different captive breeding management practices and natural histories. Results indicate lower diversity in neutral and MHC class I genes for the western population, and the opposite for MHC class II. A lower MHC class II gene variability led to an increase in microbial phylogenetic diversity and in abundance depending on the presence of specific MHC-II motifs. Those results seem to be linked to management practices that differs between the two programs, especially the number of generations in captivity.

Finally, I simulated a captive breeding program on white-footed mice for reintroduction purposes over one generation. I assessed the impacts on the gut microbiota of early-life diet change in captivity and established if a wild-like diet can foster the recovery of a wild-like microbiota once the animal in relocated from captivity to its natural habitat. Relocated mice that had the treatment diet had more phylotypes in common with the wild-host microbiota than mice under the control diet or mice kept in captivity.

Overall, our results shed light on the importance of early life colonization and the potential that diet modification has on microbial modulation to foster potentially key taxa in the gut microbiota when hosts are held in captive settings. It is likely that holding animals in captivity within the distribution area of the species could foster ancestral states, but diet should include as many natural components of the species diet as possible. Additionally, practices where sexual selection can happen should be considered. That is why the concept of holobiont is important, to bring microbiologists to the conservation biology table, to better understand our natural world.

Résumé

La biologie subit un changement de paradigme, où les phénotypes sont perçus comme un résultat d'interactions complexes de la combinaison de l'expression du génome de l'hôte et de ses microorganismes associés, menant à la popularisation du concept de l'holobionte. Les vastes communautés de microbes comme les bactéries présentes dans l'intestin des mammifères sont connues pour jouer des rôles vitaux and la croissance et la maintenance d'organismes multicellulaires sains, bien que les relations complexes entre hôte et microorganismes restent à explorer. Cela est d'autant plus remarquable chez les espèces en voie d'extinction, faisant face à des enjeux de conservation extrêmes, et qui demeurent moins étudiés que les animaux domestiques et les humains.

Les programmes d'élevage pour la conservation, comprenant des périodes de captivité et des contraintes dans le système de reproduction, peut promouvoir des changements au niveau du microbiote intestinal chez les espèces menacées. La captivité implique notamment des soins vétérinaires importants, un contact limité avec d'autres espèces et/ou des congénères, mais aussi un régime alimentaire et substrats différents par rapport aux conditions naturels des espèces. L'utilisation de médicaments comme les antibiotiques, ainsi que des changements de régime alimentaire sont connus pour impacter le microbiote intestinal des mammifères. Toutefois, le passage d'environnement sauvage à captif peut modifier le microbiote intestinal différemment chez les espèces, en fonction de leur écologie et histoire naturelle. Étudier ces changements est important pour comprendre à quelle profondeur les programmes d'élevage ont un impact sur l'holobionte, afin de promouvoir la survie et la reproduction de chaque individu, et par extension le futur des espèces.

Dans cette thèse, nous avons étudié deux classes de facteurs de variation du microbiote intestinal chez des espèces menacées dans des programmes d'élevage. D'un côté, les facteurs héréditaires qui permettent des changements potentiellement irréversibles, ou réversibles sur multiples générations de l'hôte, et opèrent donc sur une échelle à long terme. De l'autre côté, les facteurs immédiats peuvent provoquer des variations de microbiote d'une manière

réversible et au niveau individuel de l'hôte. Nous avons donc étudié ces différents facteurs: le génotype de l'hôte, l'environnement de naissance, le régime alimentaire, l'acte d'hibernation ainsi que l'environnement (captivité et géographie) afin de comprendre lesquels étaient héréditaires ou immédiats.

Dans un premier temps, la diversité taxonomique et l'abondance des communautés bactériennes de l'intestin de la marmotte de l'île de Vancouver en danger critique ont été étudiées en comparant des individus sauvages et captifs dans des institutions *in* et *ex situ*. La diversité microbienne était plus élevée chez des marmottes captives dans des établissements en dehors de leur aire de distribution naturelle par rapport aux autres congénères présents *in situ*, et des différences de composition furent observées également. Des biomarqeurs bactériens ainsi que les prédictions métagénomiques indiquaient que ces variations basées sur l'environnement géographique de l'hôte pourraient être liées à un changement de régime alimentaire, notamment en nourriture crue, entre établissements *in situ* et *ex situ*.

Nous avons aussi conduit une analyse longitudinale dans les périodes et pré- et post-hibernation et examiné les différences en microbiote intestinale entre marmottes contenus dans les établissements *in situ* et *ex situ.* Les animaux nés dans l'habitat naturel arboraient des communautés bactériennes différentes par rapport à leurs congénères nés en captivité. Dans la période de pré-hibernation, les marmottes captives *ex situ* semblent posséder une plus grande proportion de taxa qui sont métaboliquement versatiles, par rapport à une grande abondance de bactéries connues pour dégrader des mucines chez les marmottes captives *in situ* Pour la période de post-hibernation, les marmottes *ex situ* arboraient plus de taxa associés à la dégradation des fibres végétales par rapport aux marmottes *in situ.* Sachant que les marmottes *in situ* ont un microbiote intestinal similaire à leurs congénères sauvages, ces résultats confirment l'intérêt d'un transfert d'animaux captifs au zoo à l'établissement *in situ* avant leur réintroduction.

La théorie de la phylosymbiose a aussi été testée: nous avons examiné si les liens de parenté entre hôtes se reflètent par des similarités de microbiote intestinal entre populations. Le vison d'Europe endémique et le vison d'Amérique invasif en Europe de l'Ouest ont été utilisés comme modèles, et leur microbiote intestinal a été étudié en fonction de la parenté et l'environnement de leur hôte (captif ou en liberté). Nos résultats montrent des différences entre animaux captifs et sauvages, éclipsant l'appartenance aux espèces. Cependant, les visons d'Amérique sauvages d'une population bien implantée exhibent une composition bactérienne plus proche des visons d'Europe endémiques sauvages par rapport à une population sauvage plus récente de la même espèce. Ce résultat pourrait être expliqué par des variations de régime alimentaire entre les populations basées sur la disponibilité des proies entre les différents habitats, plutôt que par rapport à la différenciation génétique.

Les impacts du génotype de l'hôte (en gènes MHC et marqueurs neutres) sur le microbiote intestinal furent aussi examinés. Nous avons comparé deux populations captives distinctes du vison d'Europe en danger critique d'extinction avec deux types de gestion de programme d'élevage et histoires naturelles différents. Les résultats indiquent une plus faible diversité en marqueurs génétiques neutres et en gène MHC-I pour la population occidentale par rapport à l'orientale, en opposition au gène MHC-II. Une faible variabilité du gène MHC-II est corrélée à une augmentation de la diversité phylogénétique microbienne, et à des variations d'abondance en fonction de la présence de motifs de MHC-II spécifiques. Ces résultats semblent liés aux techniques de gestion entre les deux élevages, notamment le nombre de générations en captivité et le nombre d'animaux nés sauvages.

Enfin, nous avons simulé un programme d'élevage avec la souris à pattes blanches pour la réintroduction après une génération. Nous avons évalué les impacts d'un changement alimentaire en captivité dans les premiers stades de vie des hôtes sur leur microbiote intestinal, et si un régime alimentaire proche du sauvage peut favoriser un retour à l'état ancestral du microbiote une fois l'animal réintroduit dans son habitat, par rapport à un régime transformé (granulés de laboratoire). Les souris réintroduites ayant été nourries au régime traitement avaient plus de taxa bactériens en commun avec les animaux sauvages, par rapport aux souris réintroduites avec le régime transformé, ainsi que les souris en captivité.

Globalement, nos résultats apportent de nouvelles preuves sur l'importance de la colonisation microbienne dans les premiers stades de vie, et sur le potentiel qu'a le changement de régime alimentaire sur le microbiote intestinal, afin de favoriser des taxa importants lorsque les hôtes sont contenus en captivité. Le fait que les animaux soient détenus en captivité à l'intérieur de leur aire de répartition pourrait favoriser les états ancestraux (sauvages) du microbiote, mais il est important que le régime alimentaire contienne le plus possible d'aliments naturels pour cette espèce. De plus, des pratiques où la sélection sexuelle peut avoir lieu devrait être considéré afin de favoriser le brassage génétique des hôtes et par conséquent un microbiote sain. C'est pourquoi le concept de l'holobionte est important, afin de rassembler les microbiologistes à la table de discussion de la biologie de la conservation, nous permettant de mieux comprendre le monde dans lequel nous vivons.

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Chapter 1. Introduction

1.1 The intricate world of mammalian microbial communities

1.1.1 A new field to explore

The holobiont concept is the view that animals are not autonomous entities, but rather holobionts - the host together with its associated microbes (Bordenstein & Theis, 2015). The majority of genes in the holobiont system are of microbial origin. These microbes interact with their host in many ways and can have a pathogenic, mutualistic or commensal role. Early studies of host-associated microbes were challenged by the limitations that arose from culture-dependent methods that failed to account for microbes that are to date difficult to isolate and culture (Lagkouvardos et al., 2017). With the extensive development of molecular techniques (e.g. genetic information extraction, NGS sequencing) in the last twenty years, we are beginning to explore the intricate relationships between hosts and their microbes, firstly in humans (Hamady & Knight, 2009; Song et al., 2018). Despite being relatively recent, the field of microbiome research can shed a new light on many biological processes, from human medicine, ecosystem functioning and conservation of endangered species (Carthey et al., 2019).

The microbiota is defined as the entire community of microbes present in a specific body part of a living host. Although Bacteria, Archaea, viruses, Protozoa and fungi are part of the microbiota, I will mostly refer to the bacterial communities in this thesis. Most microbiome studies, as these described in this thesis, focus on the bacteriome, defined as the bacteria and their associated genes in a microbial community. It represents the most important biomass in the gut microbiome and their abundance is higher than the other microbial taxa (Huffnagle & Noverr, 2013).

Beginning with ancient associations and continuing throughout time in a massive coevolutionary process, microbes shape their host's biology in many ways. For example, the microbiota has an influence on the immune system and the metabolism of the host and thus has consequences on the host's survival and reproduction (Spor et al., 2011; Barhndorff et al., 2016; Rosshart et al., 2017; Suzuki, 2017; Gould et al., 2018). The microbiome, defined as the presence of microbial species, their abundance and their genomes, is associated with the host's form, function, fitness, behaviour and sociality (Ezenwa et al., 2012; McFall-Ngai et al., 2013). Unlike an open environment, one must consider that the host is also subject to selective pressures that shape microbial dissemination (Ley et al., 2008). For instance, a host with poor fitness will be less likely to transmit its microbiota through reproduction and social

interaction. The host can also select bacterial communities through its immune system, dietary shifts, changes in morphology, and kinship to a lesser extent and this will all be referred to as host control (Foster et al., 2017). In that way, both the host and the microbiota shape each other through long- and short-term processes. In addition, microbes interact with each other and shape their community.

1.1.2 Acquiring and shaping microbial communities

There are many ways the host can acquire microbes. Transmission first takes place vertically during birth, where the offspring acquires the mother's microbial community (Colston, 2017). In mammals, offspring acquire the microbes from the mother's vaginal microbiota during birth and through milk consumption (Spor et al., 2011). Another theory postulates that microbes from the mother's placenta could be transmitted to the offspring during gestation, but this has not been proven experimentally (Funkhouser & Bordenstein, 2013; Koskella et al., 2017). The second and largest part of microbiota acquisition is horizontal and depends on the ecology of the host. It includes associations with conspecifics and other species, dietary sources, environmental sources (substrates, water) and coprophagy (Colston, 2017). The microbiota of juveniles is highly dependent on all these factors, and it varies until individuals enter adulthood and achieve a sufficient level of stability and resilience to overcome a variety of stressors such as exposure to diseases, pollution and food shortage (McFall-Ngai et al., 2013; Moloney et al., 2014).

In the case of the gut microbiota, it is generally accepted that the microbial community's structure, composition and heredity are more closely linked with diet than host phylogeny, even in the same species (Ley et al., 2008; Muegge et al., 2011; Phillips et al., 2012; Van Opstal & Bordenstein, 2015). Diet can have substantial impacts on the microbiota at both daily and evolutionary scales (McFall-Nagai et al., 2013). This has been demonstrated through the evolution of human lifestyles. Human microbial communities have been shaped through changes from hunter-gatherer and nomadic societies to farming, sedentary and urban lifestyles (Cho & Blaser, 2012). Common characteristics of modern Western human lifestyles, such as spending high proportions of time indoors, antibiotics and caesarean birth, have resulted in a reduction in gut bacteria diversity that has been implicated in many diseases (Segata, 2015; Zinöcker & Lindseth, 2018; Sonnenburg & Sonnenburg, 2019). On a short temporal scale, maintaining mammal species in captive settings such as zoos can lead to similar outcomes as modern Western human lifestyles, yet microbiota changes have been shown to be reversible with dietary shifts. In mice, the gut microbiota can bear major changes after an average of 3.5 days after a dietary shift (Carmody et al., 2015). Diet, therefore, plays a dominant role in shaping the gut bacterial communities at both long- and short-term scales.

Each microbiota of a host must be viewed as an open ecosystem. The physical and chemical conditions are provided by the host and vary according to the interactions between the different species and communities of microbes (Donaldson et al., 2015). Like other ecosystems, there are complex food webs, including competition for nutrients between taxa, mutualism, invasions and extinctions (Berg et al., 2020). However, classical theories in ecology cannot always be applied as they are mainly based on eukaryotes. Bacteria exhibit different mechanisms of evolution, such as horizontal gene transfer, that are not fully understood and complex to study, but accelerate the variation of specific and functional diversity. In the same way, quorum sensing (the stimuli and responses of microbes in high bacterial density), cross-feeding (a relationship where one organism consumes the metabolites produced by another), and feedback mechanisms between the microbiota and the host are not considered in currently accepted theories in ecology and evolution that generally apply to macro-organisms (Koskella et al., 2017).

The host provides the physical conditions and the necessary resources for the microbes to survive. On the other hand, the microbiota provides the host with expanded metabolic capabilities such as additional energy and nutrient extraction from their diet (McKenney et al., 2018). The gut microbiota reduces the invasion of pathogens, can detoxify harmful compounds for the host and allows the degradation of complex dietary components into molecules that the host can directly assimilate. Those functions can be referred to as ecosystem services towards the host. Each body part of an individual can follow the theory of island biogeography (Costello et al., 2012) as well as local diversification, natural selection and dispersal, borrowed from macro-community ecology. Likewise, the gut bacterial communities mainly depend on what the animal ingests through its digestive tract. Food intake provides the largest energy source for microbial growth. The gut microbiota plays an important role in animal nutrition, especially in carbohydrate fermentation (Flint et al., 2012; Krajmalnik-Brown et al., 2013). Through fermentation and microbial degradation, the dietary fibres in the intestines are broken down into oligo- and monosaccharides in the case of herbivore hosts. They will undergo degradation by the same or other bacteria, secondary degraders into Short Chain Fatty Acids (SCFAs). Through the degradation process, many secondary metabolites will be produced by the bacteria that can be used by the host or other bacteria. Those metabolites are all the molecules that do not take part in the assimilation of nutrients and can be toxins, antimicrobial peptides, signaling molecules for quorum sensing or protection/reparation mechanisms against DNA degradation. The SCFAs can then be assimilated by the host and the gut bacteria. Some gut bacteria can also assimilate endogenous substrates from the host such as mucus glycans, secretions from the pancreas and nutrients in epithelial cells (Carey & Assadi-Porter, 2017).

1.2 Gut microbial communities in the context of species conservation

1.2.1 Captivity as a driver for microbiota variation

One quarter of mammal species face extinction and therefore so do their associated microbes (Ceballos et al., 2005), and understanding the relations between endangered mammals and their microbiome is necessary for species survival, as microbe community variation intricately influences host's fitness (Suzuki, 2017; Gould et al., 2018). Many studies have focused on how diet affects the gut microbiota of mammals, but most of the research has been carried out on laboratory animals, over short periods of time, and in relation to diseases or administration of antibiotics (Moloney et al., 2014). This is particularly relevant for critically endangered animals in conservation breeding programs. Their populations are usually divided between wild, captive and reintroduced individuals. It is imperative that conservation managers retain the biological integrity of the species throughout the period in a captive setting. Captivity of mammals in zoos usually involves highly restricted diets, closed and small habitat size, lower social interactions and exposure to medicine like antibiotics. Mitigating the effects of captivity is critical to limiting the evolution of 'unnatural' populations that can negatively affect survival of released animals, or even domestication. One endangered species is the Vancouver Island marmot (*Marmota vancouverensis*), the host in the second and third chapters. The Vancouver Island marmot is a hibernator that undergoes cycles of fasting that reflect natural extreme diet changes. Understanding microbial ecology, the native microbiota and their link with diet is needed, especially in the context of captive breeding for hibernators. Approaching species conservation from a systemic point of view such as the holobiont concept can bring new insights and strategies to foster species survival.

The consensus is that captive animals tend to have lower gut microbial diversity and a different microbiota composition compared to wild animals, mostly in the Firmicutes and Bacteroidota phyla that represent the majority of mammalian gut microbes (Kohl & Dearing, 2014; McKenzie et al., 2017). However, McKenzie et al. (2017) observed different trends, and captivity did not have the same effect on the gut microbiota investigated. Microbial alpha-diversity (bacterial species richness) is variable depending on the species, whereas beta-diversity (variation in community composition) is mostly observed to be different between captive and wild animals across species (West et al., 2019). Patterns are already emerging in this recent area of research. Two studies suggest that animals that are considered as specialists, with a very strict diet, tend to exhibit gut microbiota close to the wild populations (Kohl et al., 2014; Alfano et al., 2015). This trend is not supported in all species, such as the red panda (*Ailurus fulgens*), although some microbial taxa were similar between wild and captive animals (Kong et al., 2014). A study on Przewalski's horses (*Equus ferus przewalskii*) examined variation in composition of the microbiota among animals born in zoos, reserves and in the wild. Horses born in captivity had a lower diversity of fecal microbiota compared to horses born in a reserve (Metcalf et al., 2017). Hence, we hypothesize that anthropogenic forces can shape

the animals' associated microbes, which can have implications in the management of endangered species. Captivity associated traits may persist in zoo-born animals and could have consequences that will impact the success of the reintroduction programs and therefore the survival of the species in the wild (Carthey et al., 2019). Relationships between the microbiota, diet and captivity therefore need to be further investigated. By conducting an experiment on free-ranging white-footed mice (*Peromyscus leucopus*) in the sixth chapter, I simulated a captive breeding program over one generation to study the impacts of captive diet during the translocation process in the natural habitat. I aimed to investigate whether a standardized transformed diet or a whole foods easily accessible version of a wild-like diet could mimic to an extent a wild mice microbiota that never underwent captivity.

Few studies to date have examined the effect of captivity on the functional diversity (range of ecological niches within an ecosystem) of the gut microbiota of mammals. Microbial genes involved in metabolism have been demonstrated to be largely similar between wild and captive individuals apart from xenobiotic degradation gene families (Borbon-Garcia et al., 2017). This could be linked to differences in environmental settings and the presence of opportunistic bacteria acquired from the wild due to more varied access to plant microbiota that developed defense mechanisms against herbivores. I want to verify this hypothesis at the species level between captive held in *ex situ* and *in situ* facilities and wild populations of the Vancouver Island marmot. Are there differences in gut microbial communities in marmots held at different facilities? And if so, which one is closer to wild conspecific and what could be the reason? I will explore those questions in the second chapter. A study on captive and free-ranging cheetahs showed that captive animals had a microbiome with an increase in gene content taking part in immune system and neurodegenerative diseases (Wasimuddin et al., 2017). Functional diversity may be particularly informative in describing environmental drivers of the microbiome.

1.2.1 Host genotypes are also at play

As previously described, the composition of the microbiota is affected by many environmental factors depending on the region of the body where it is located (Donaldson et al., 2015; Spor et al., 2011). The gut bacterial communities can be influenced by diet, use of antibiotics, birth, social interaction and the general environment of the animal (Amato, 2013; Barhndorff et al., 2016). To a lesser extent, the host's genotype can also influence the microbiota acquisition and therefore composition in vertebrates (Spor et al., 2011; Koskella et al., 2017; Rothschild et al., 2017).

One of the ways that genetic background can influence gut microbial composition is explained through the phylosymbiosis theory. Phylosymbiosis is described as an increase in

compositional similarity between bacterial communities colonizing closely related hosts compared with distantly related hosts (Groussin et al., 2017; Lim & Bordenstein, 2020). Many investigated mammals have supported this pattern, such as bats, apes and rodents (Brooks et al., 2016; Ochman et al., 2010; Kohl et al., 2018; Knowles et al., 2019), as well as other animal taxa (Pollock et al., 2018; Sevellec et al., 2019; van Opstal & Bordenstein, 2019); however, other studies have not detected phylosymbiosis (Baxter et al., 2015; Greene et al., 2019; Grond et al., 2020). Groussin et al. (2017) also suggested that the associations between some host taxa and some of their associated gut microbes might not generalize to the entire gut microbial community, hence the strong environmental effects on gut microbial composition. No study to date has examined phylosymbiosis in the context of invasion ecology in carnivores. Carnivores have short transit time and digestive tracts, so the gut microbiota are potentially less impacted by diet (Reese & Dunn, 2018; Ley et al., 2008). I therefore attempt to fill the gap in the literature by studying gut microbial variation between two related carnivores, the native and endangered European mink (*Mustela lutreola*) and the invasive American mink (*Neovison vison*) in Western Europe in the fourth chapter, across captive and free-ranging environments.

As endangered species are in most cases reduced to small isolated populations prone to loss of genetic diversity, a key challenge of captive breeding programs is to maintain genetic diversity and avoid inbreeding depression with a small number of founders (Bouman, 1977; Ralls et al., 1979). At the intra-species level, genes involved in immune responses are known to control microbiota composition, for example NOD2 and IL-23R, and Single-Nucleotide Polymorphisms (SNPs) (Koskella et al., 2017). Within this context, Foster et al. (2017) proposed a theoretical framework known as the ecosystem on a leash model, which posits that the host is under strong selection to evolve mechanisms to keep the microbiota under control. The presence of a genetically diverse microbiota leads to the dominance of the fastest growing microbes instead of the microbes that are most beneficial to the host (Foster et al., 2017). The targeting of microbial taxa to either limit their proliferation could thus be beneficial to the host, through its adaptive immune response. The major histocompatibility complex (MHC) genes play a key role in immune recognition. The proteins produced bind to antigens to trigger T-lymphocytes responses (Potts et al., 1994). MHC is considered the most polymorphic loci known in vertebrates (Sommer, 2005). The genetic diversity of MHC is a result of selection from pathogens, inbreeding and mate selection (Radwan et al., 2010). Mate selection aims to reduce homozygosity with deleterious alleles and the presence of rare genotypes through frequency-dependent variation. In the same way, no inbreeding would favor heterozygosis. Few studies have focused on the impact of MHC allelic diversity on the microbiota (Amato, 2013). Bolnick et al. (2014) demonstrated that certain motifs in MHC class II were associated with the presence of bacterial taxa in fishes and that most diverse motifs resulted in reduced microbial species richness. However, no studies to date have investigated

this link between host genotype and gut microbiota within the context of conservation breeding programs. In this extreme context, I therefore hypothesize that less host control, expressed by more genetically diverse gut microbes, should happen in individuals with reduced genetic diversity in both neutral and adaptive markers. To test this hypothesis, I investigated in the fifth chapter the genetic diversity and gut microbial community assemblages in the critically endangered European mink.

1.3 Studying microbial communities

A healthy ecosystem, and more particularly a healthy microbiota, is mainly characterized by a high and stable diversity. Bacteria produce metabolites that play a role in the ecosystem functioning of the host. Many pathways and mechanisms of actions of these metabolites remain unknown due to the difficulty of isolating and culturing the responsible bacteria. It is also challenging to recreate the conditions in which one organism produces the metabolites; either with specific physical conditions and/or the potential mandatory presence of other microbes (Hofer, 2017). If we consider the gut microbiome as an ecosystem, the host's health can be seen as an ecosystem service from this microbiome. We can therefore apply the established framework to manage ecosystem services from applied ecology (McKenney et al., 2018).

The first step is to identify the taxa present in the microbiome ecosystem. Culturing microorganisms from mammalian gut samples has been proven to be challenging. Most of the gut microbiota do not grow under laboratory conditions, which are widely different for the gut of animals which is why NGS techniques are mostly used in this type of study. In this way, the use of metabarcoding is less costly to study bacterial communities compared to metagenomics. This method involves DNA extraction for the biological samples collected. This study will be using fecal samples, which are accepted as a good representation of the gut microbiota (Goodrich et al., 2014; Kohl et al., 2017). After DNA extraction for these samples, the targeted genes for taxonomic affiliation are amplified through Polymerase Chain Reactions (PCRs). The widely accepted 16S rRNA gene is considered as the golden standard for bacteria, and at the same time for Archaea. Once the microbial DNA is amplified and libraries prepared, it is sequenced on an Illumina MiSeq or NextSeq platform. The quality controls of the sequence reads will be performed through the QIIME2 pipeline (Caporaso et al., 2010), as well as the Operational Taxonomic Units (OTUs) picking and clustering. OTUs, also referred to as phylotypes, provide a proxy of name groups of related bacteria. They are based on sequence identity to reference sequences. Depending on the percentage of resemblance to those reference sequences, we can cluster OTUs to different taxonomic levels. With 95% of similarity, an OTU can be assimilated to a genus and with 97% to a species, although those thresholds are still discussed (Goodrich et al., 2014).

When the taxonomic classification is complete, the second step is to investigate which ecosystem functions the microbial communities bring to the system. To explore functional diversity in the microbiota, I used the program PICRUSt (Langille et al., 2013). This program uses the 16S rRNA gene marker to predict the metagenomic content of a microbial community through available sequence inventories. This program produces a chimeric metagenome for each known OTU to the closest relative with a reference genome. The abundance of bacterial reference genomes from endangered mammalian gut is scarce and horizontal gene transfers are not measurable by this technique. It will therefore not completely reflect the functions from the original sample and conclusions should not be drawn without conducting metagenomic or metatransciptomic approaches. However, it is a costless and useful tool to investigate the gut microbiome. Within this framework, I aim to get a sense of which taxa are present in the gut communities, their functions, and try to explore the environmental drivers operating on the microbiome.

After establishing what taxa are present in the ecosystem and their putative functions, we can hypothesize how the ecosystem functions. Finally, we will evaluate how the communities influence ecosystem functioning and identify environmental drivers modulating the ecosystem at different temporal and spatial scales on the ecosystem's functions (Costello et al., 2017). This will be done through statistical analysis by comparing taxonomic variation using alpha, beta diversity indices as well as differential abundance analysis between samples under different conditions.

I postulate that the composition of the gut microbiota is influenced by two broad classes of drivers (Moeller & Ochman, 2013). Firstly, heritable drivers are potentially not reversible, or if so, over multiple host generations, therefore operating at long-term scales. I hypothesize that host genotype and birth location are heritable drivers. Second, immediate drivers could induce variation in microbial community composition in a reversible manner and at the individual level of the host. I therefore hypothesize that host diet, biology (such as hibernation) and environment (captivity and geography) are short-term immediate drivers. Overall, I hypothesize that long term dietary shifts (over more than one generation of the host) should be a bridge between the short scale variation of the microbiota composition and its long-term evolution. This hypothesis is supported by the nutrient niche theory, which states that limiting nutrients control the species diversity and abundance that can process them and therefore their functional diversity (Donaldson et al., 2015). The overall objectives in this study are to test this framework and to understand how environmental drivers of the host intervene in this complex setting.

1.4 Overview of the dissertation

This doctoral project is carried out in the framework of the ReNewZoo research program funded by the Natural Sciences and Engineering Research Council of Canada (NSERC). ReNewZoo is a graduate (MSc/PhD) training program that brings together zoos/aquariums with academic ecologists/conservation biologists. This project was also conducted jointly with the Boreal Ecology PhD program of Laurentian University (Canada) and the Ecole doctorale de biologie cellulaire et moléculaire, bioinformatique et modélisation from Université de Liège (Belgium).

As endangered species face many conservation challenges, the main goal of the present PhD study is to investigate threats (loss of genetic diversity, apparent competition with an invasive species, hibernation-related mortality) and to study their impact on the animal's gut microbiota. Both the European mink and Vancouver Island marmot are part of captive breeding programs, and therefore provide a framework in which to study the impacts of captivity on the microbiome. This will be investigated from the environmental perspective: the comparison between in situ and ex situ facilities (1), and in relation with the host's biology: how the microbiota is modified by hibernation (2) or if host genetics has a strong impact on it (3), and testing the phylosymbiosis theory (4). I finally tested the impact of diet manipulation (5), and the transition from captivity to the animal's natural habitat (5). Studying all these aspects will allow a better understanding of the host-microbial relationships and may be helpful for conservation biologists in the decision-making process to maximize reintroduction success.

This thesis is divided into seven chapters.

Chapter 1 is a general introduction for the study.

In Chapter 2, the taxonomic diversity and abundance of bacterial communities of the Vancouver Island marmot's gut in the wild compared to *in situ* and *ex situ* facilities were investigated. I examined the potential influence of sex, age, genetic relatedness, source of mortality and change in environment on gut microbial communities during the active season in captive and free-ranging Vancouver Island marmots, a critically endangered herbivore that undergo hibernation.

Chapter 3 presents the taxonomic and potential functional variation of gut microbiota from the Vancouver Island marmots in captivity. I conducted a longitudinal analysis in the pre- and post-hibernation periods and investigated the differences among marmots held at the *in situ* and *ex situ* facilities.

In **Chapter 4**, the phylosymbiosis theory was tested: I examined if host phylogenetic relatedness correlates with gut microbial communities' similarities between populations. The native European mink and the invasive American mink in Western Europe were used as models in this chapter, and their gut microbiota was studied according to their relatedness and environment (captive or free-ranging).

The impacts of host genotype (in MHC and neutral markers) on gut microbiota were investigated in **Chapter 5**. I compared two distinct captive populations of the critically endangered European mink with different captive breeding management practices and natural histories.

In **Chapter 6**, I simulated a captive breeding program on white-footed mice for reintroduction purposes over one generation. I assessed the impacts on the gut microbiota of diet change in captivity and established if a wild-like diet can foster the recovery of a wild-like microbiota once the animal in relocated from captivity to its natural habitat.

Finally, **Chapter 7** presents the general findings of this thesis and a general conclusion.

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Chapter 2. Bacterial community variation in the endangered Vancouver Island marmot (*Marmota vancouverensis*) under *in situ* and *ex situ* captive breeding programs and in its natural habitat.

2.1 Summary

Although the intricate link between gut microbial communities (GMCs) and host fitness/survival is well acknowledged, the influence of host traits and environmental factors on GMCs in wildlife is variable depending on the host studied. Such information is important within the context of conservation biology, especially when hosts are under captive management and are often translocated between environments. Through 16S rRNA amplicon

sequencing, I examined the potential influence of sex, age, genetic relatedness, source of mortality and change in environment on GMCs during the active season in captive and free-ranging Vancouver Island marmots (*Marmota vancouverensis*), a critically endangered herbivore that hibernates. Gut microbial diversity was higher in marmots held in *ex situ* facilities, outside of their habitat range, compared to captive marmots held within their habitat range, and in the wild and differences in composition were also observed. Microbial biomarkers (*Gastranaerophilales*, *Oscillospiraceae*, *Lachnospiraceae* enriched in *ex situ* marmots) and metagenome predictions indicated that those variations could be linked to diet change in raw foods between *in situ* and *ex situ* facilities. Other biomarkers were also identified that could help prevent marmot mortality in captivity. Overall, this study provides a first in-depth look into the GMCs of hibernating mammals that offers knowledge for *in situ* and *ex situ* conservation programs.

2.2 Introduction

The Vancouver Island Marmot (VIM; *Marmota vancouverensis*, Swarth 1911) is a colonial marmot and one of only five endemic mammalian species in Canada. This species is restricted to Vancouver Island, British Columbia, in small and widely scattered alpine meadows, between 1000-1400 m elevation (Armitage, 2000). Their diet is composed of approximatively 50 species of grass, herbs and wildflowers (Milko, 1984). Like other species of marmot, VIM are social mammals and depend on burrow systems to hide from predators, reproduce and hibernate in groups (Armitage, 2000). They have annual cycles of hibernation periods from October to early May and active periods during the rest of the year (Armitage, 2000). Their sociality is mainly restricted to the family level, composed of one male and one to three females. Females have on average one litter per year and the young disperse at 2 years of age (Casimir et al., 2007).

This species has been classified as endangered since 1982 and critically endangered since 2008 by the IUCN (Roach, 2017). The proximate cause of decline is the excessive predation of marmots due to landscape change from logging. Predation by species like golden eagles, cougars and grey wolves has been estimated to be the cause of 50 to 80% of marmot mortality (Bryant & Page, 2005). In the late 1990s, the overall free-ranging VIM population was estimated at 70 individuals with a drop to approximately 30 individuals by 2007 (VIMRT, 2017).

With the implementation of a captive breeding and reintroduction program, the marmot population increased to approximately 300 marmots in 2013 with a steady decline since then. In 2019, there were estimated to be around 200 individuals in the wild and 53 in captivity (VIMRT, 2020). The captive breeding program involves three separate locations: two *ex situ*

facilities (Toronto Zoo and Calgary Zoo) as well as one *in situ* facility (Tony Barrett Mount Washington Marmot Recovery Centre, MRC). *In situ* is defined as on-site conservation within Vancouver Island, whereas *ex situ* refers to off-site locations, outside of the animal's geographical range. Zoos have thus played an important role in the conservation of this species as they play an active role in the VIM recovery program, trying to maximize genetic diversity through selective breeding and offering a predation-free environment. Every year, captive-bred marmots are transferred to the MRC on the island to be acclimated to their native habitat and then released into existing colonies. Captured wild-born VIM also hibernate at the MRC and either breed there or in one of the *ex situ* locations. From 2000 to 2017, 490 captive-bred marmots were released (VIMRT, 2017), which is more than twice the number of established marmots on the island, showing intense conservation efforts and low survival rate of released marmots.

In addition to predation, it has been shown that captive-born marmots experience low overwinter survival compared to wild marmots (Jackson et al., 2016). Since 2009, the overwinter survival of captive-released marmots in their first winter has been lower than 40%. A recent study showed that captive marmots hibernated for shorter periods than wild-born marmots, and increased body weight was associated with hibernation length, potentially indicating variation in metabolic rates between wild and captive VIM (Aymen et al., 2021). In captivity, few mortalities have been observed since 1997 (8%), the first cause being cardiovascular complications (22%), infectious/inflammation and hibernation related mortalities being second (18.3%), followed by neoplasia (16.3%, VIMRT,2020).

The conservation concern and behavioural ecology of the VIM makes this species an interesting model to investigate the impact of host traits and external factors on its natural gut microbial range. It is recognized that Gut Microbial Communities (GMCs) differ within mammalian host species between free-ranging and captive conspecifics. However, variations occur in different ways (McKenzie et al., 2017, reviewed in West et al., 2019). An increase in gut microbial diversity (alpha diversity) was marginally observed in captive hosts such as tigers, Rhinocerotidae and primates (McKenzie et al., 2017; Campbell et al., 2020; Ning et al., 2020), while many studies documented a decrease in alpha diversity in numerous captive mammals compared to free-ranging conspecifics. This decrease has been associated with decreased dietary diversity, antibiotic use, and increased sanitation. An alteration of fiber digestion related microbial function was also observed, especially for herbivores (Amato et al., 2016; Clayton et al., 2016; Borbon-Garcia et al., 2017). In herbivores, the GMCs are involved in the breakdown of fibrous plant material into various metabolites including Short Chain Fatty Acids (SCFAs) that have a significant impact on host metabolism, and many other beneficial functions (Dearing & Kohl, 2017). Microbial variation in composition has also been correlated with host diseases within captive settings (Krynak et al., 2017). Overall, these GMCs

alterations could potentially reduce the success rate of translocation and reintroduction by impacting host survival, especially in an herbivore like the VIM that undergoes a critical period that depends on metabolic efficiency like hibernation (Carey & Assidi-Porter, 2017; Lindsay et al., 2020).

Few studies to date have examied GMCs variation among animals present in wild habitat, and *in situ* and *ex situ* facilities within captive breeding programs. Webster et al. (2011) observed greater stability in GMCs for marine sponges for *in situ* cultivation sites compared to *ex situ*, but the definition of captivity might not apply in this context because the sponges were secured to the reef in open water and not in an aquarium. I argue that holding animals in captivity within their geographical range might offer greater opportunities for microbial transmission from historical substrates, as well as reduced variation in abiotic conditions such as photoperiodism that might mitigate metabolic changes, influencing circadian rhythms and hibernation (Ren et al., 2020). In the long run, GMCs manipulation has been suggested to enhance host survival for animal conservation, potentially through diet and prebiotic modification, as well as probiotic supplementation (West et al., 2019).

Before attempting any microbiota engineering or diet manipulation, critical first step is to describe the baseline GMCs within the VIM context and explore how they fluctuate. This study focuses on the gut microbial diversity within the active season. I applied a 16S rRNA gene high-throughput sequencing approach to fecal samples of VIM to investigate the influence of sex, birth environment, presence of pups, outside access, genetic background (i.e. relatedness) and location (free-ranging/*in situ*/*ex situ*) on their gut microbiota diversity and associated putative metabolic functions. GMCs were also investigated according to source of mortality for captive animals that died during the study. Further, I studied the variation of the GMCs in two longitudinal scenarios: when wild VIM are captured and translocated to a captive environment, and when VIM are moved from *in situ* to *ex situ* locations.

I expected strong effects of the environmental factors on microbial diversity but not of the intrinsic factors, especially between free-ranging and captive VIM, as those animals face extremely different biotic and abiotic conditions. I also hypothesized that alpha diversity would be lower in captive VIM, both *in situ* and *ex situ,* compared to free-ranging conspecifics, as this pattern is largely observed in other herbivores. Furthermore, I expect to find a greater abundance of microbial taxa related to plant degradation in free-ranging VIM compared to more metabolically versatile taxa in captive VIM due to diet variety in captive settings (pellets, raw foods).

2.3 Materials and methods

2.3.1 Sample collection and information

All methods were approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University and by the Toronto Zoo Animal Care and Research Committee (ACRC; 2018-05-02). A total of 176 fecal samples were collected from 86 individuals and pairs of VIM for this study in 2018 and 2019 from three separate locations: the *ex situ* (Calgary Zoo and Toronto Zoo) and *in situ* facilities (Tony Barret Mount Washington Marmot Recovery Center – MRC), and in the VIM natural habitat on Vancouver Island (Table 1). While free-ranging marmots were sampled individually, VIM in captive housing facilities are paired for mating and pup rearing throughout the year in their enclosures. Since animals sharing enclosures usually defecate in the same area, it was not possible to distinguish which animal the fecal sample originated from, and those samples were therefore treated as belonging to the group of VIM present in the enclosure. Fecal samples were collected opportunistically from traps for live animals for free-ranging VIM, and during daily enclosure cleanings for captive animals using gloves. Samples were stored in sealed plastic bags in a –20°C freezer until DNA extraction. Because trapping free-ranging marmots in remote conditions is challenging and depended on presence of fecal matter in traps, only one sample per individual was possible. Sample collection in housing facilities was thus conducted accordingly to sampling dates from Vancouver Island, including two to five separate samples from the same VIM pair or individual, ranging from July 17th to September 18th, representing the mid-period of the active season of the VIM.

To gain more insight into GMCs variation when VIM are translocated between locations, I was able to sample individuals that underwent translocation within one day to a year after the move. Twelve free-ranging VIM trapped and sampled on Vancouver Island were taken into the MRC for supplementing the breeding stock and were also sampled after capture. In the same way, three male VIM were captured from Vancouver Island for transfer to the *in situ* facility and then to the Calgary Zoo, and fecal samples were collected throughout the process to investigate GMCs variation when animals are translocated between locations.

A wide range of information associated with each VIM sampled was available through the Vancouver Island Marmot Recovery Team (VIMRT) and the Zoological Information Management Software (ZIMS). Relatedness coefficients were inferred from studbook information, as well as individual sex, age, previous location and date of transfer, place of birth and birth location for each VIM's dame and sire. Source of mortality was included in the study if the individual died between July 2018 and December 2020. Three VIM died because of respiratory or microbial infection causes and two from neoplasia in captivity, while ten died from unknown reasons in the wild. For captive animals, outside access and the presence of pups in the enclosure were also documented according to sampling date. Data for

paired VIM were combined for a number of variables: the minimal age of a group, sex (M/F if pair of the two sexes; M if only males present in enclosure), and locations for place of birth for each individual, dam and sire. For example, if an animal's dame was born in the wild and sire in an *ex situ* facility, the output would be "ex situ and wild".

2.3.2 DNA extraction and sequencing

Gene amplicon sequencing was used to study the bacterial communities. DNA extractions from stored fecal samples were conducted using the Stool DNA Isolation Kit (Norgen Biotek Corp) following the manufacturer's instructions. Twelve blank extractions were made to control for contamination during the extraction process. A mock community sample (ZymoBIOMICS™ Microbial Community DNA Standard) containing genomic DNA from eight bacterial strains, at 12 ng/µl was also added in the library to confirm the reliability of our method. After DNA extraction, the targeted gene for taxonomic affiliation (16S rRNA gene) was amplified through PCRs, with one negative control added for sequencing. The library preparation and sequencing were performed by Genome Québec Inc., as well as the demultiplexing of the sequence reads. Using their designated library protocol, 2 × 250 bp with 30,000 reads/sample sequencing was completed using broad bacterial primers of the region V4 of the 16S rRNA gene (515F-806R) using an Illumina NextSeq platform (Illumina Biotechnology Co.).

2.3.3 Bioinformatics

The quality controls of the already demultiplexed paired-end sequence reads were performed through the software FastQC (Andrews, 2010). Sequence reads denoising and amplicon sequence variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al., 2018; v. 2019.1), using the DADA2 pipeline (Callahan, Mcmurdie, & Holmes, 2017; Callahan et al., 2016) with trimming forward reads to a minimum of 200bp and reverse reads to a minimum of 210bp based on quality scores. ASVs—or also referred to as bacterial phylotypes—were then screened using a pre-trained Naïve Bayes classifier on weighted Silva v.138 99% OTUs full-length sequences (animal distal gut trained dataset, Kaehler et al., 2019) for taxonomical association using the q2-feature-classifier implemented in QIIME2 (Bokulich et al., 2018). Sequence alignment and phylogeny building were also conducted in QIIME2. The mock community sample was removed from the dataset for analysis, after correct identification of 7/8 bacterial strains to the genus level (8/8 family level).

After data importation in R v.4.0.3 (R Core Team, 2018) using the phyloseq package (McMurdie & Holmes, 2013), 33 potential contaminants were identified from the extraction blank from the prevalence-based method using the Decontam package (Davis et al., 2018).

Those 33 ASVs were removed from the dataset, as well as extraction blank samples, and sequences assigned to mitochondria and chloroplasts for downstream analysis. Variance stabilizing transformation in DESEq2 was conducted on the ASV abundance table instead of sequences rarefaction for beta diversity and differential abundance analyses (Love et al., 2014; McMurdies & Holmes, 2014).

2.3.4 Statistical analysis

Faith's PD, Shannon indices and observed number of ASVs in each sample were used as metrics to measure the α-diversity of gut bacteria between samples. Differences in the index values according to VIM location, place of birth, sex, age, previous location, parents' birthplace, source of mortality, presence of pups, and whether there was outside access, were investigated using restricted maximum likelihood fitting linear mixed-effects models (lmer) with VIM pair/individual and month of sample collection as random effects. The significance cutoff was set to p-value<0.05 for each test. Homogeneity of variance assumptions were tested using Levene tests and normality of the residuals with Shapiro-Wilk tests as well as visual representations. For investigation of alpha diversity variation during translocation, the same analysis was conducted with the variables VIM environment at sample collection date and number of days after translocation.

Beta diversity was measured through weighted UniFrac distance matrices between samples (Lozupone et al., 2010). They were used to investigate differences in GMCs between the variables VIM environment, sex, previous location, parents' birthplace, source of mortality, and presence of pups, using PERMANOVA models Adonis from the *vegan* package were constructed with 9,999 permutations with reported F, R^2, and p-values (Oksanen et al., 2019), and variables with R^2<1% were dropped of the models. Pairwise permutation-based tests of multivariate homogeneity of group dispersions were then conducted to investigate variations between groups with 9,999 permutations, as well as pairwise PERMANOVAs. For investigation of beta diversity variation during the two translocation events (Vancouver Island to *in situ* facility; *in* to *ex situ* facilities), the same analysis was conducted with the variables VIM environment at sample collection date and number of days after translocation. Principal component analyses (PCoA) using weighted Unifrac distance measures between samples was conducted to visualize the dissimilarities between groups. Additionally, the turnover (Simpson dissimilarity index) and nestedness (nestedness-resultant fraction of Sorensen dissimilarity) components were calculated in the R package betapart (Baselga & Orme, 2012).

A Mantel test was also conducted to investigate correlation between GMCs composition (weighted Unifrac distance matrix) and relatedness between VIM pair/individuals. The

relatedness coefficient matrix was directly inferred from the kinship coefficient between each individual VIM derived from the studbook. Given two pairs/groups of marmots P1 and P2, the relatedness was defined as the mean kinship coefficient over all possible pairings between VIM of P1 and P2. If we consider two fecal samples, each generated by the same marmot for P1 and P2, I made the assumption that the fecal sample could have been generated by any of the member of the group with equal probability. Then, the defined relatedness was inferred between marmots that have generated each fecal sample.

Finally, I used linear discriminant analysis in effect sizes (LEfSe; Segata et al., 2011) to identify biomarkers from specific groups for the significant variables of the PERMANOVA model. Only group variables with number of VIM pair/individual>3 were included in the analysis for statistical power, and only logarithmic LDA scores above 3 with p-value<0.05 were considered significant. I then examined differential abundances in ASVs for the two translocation events datasets using the R package DESeq2 and at a significance level of 0.01, and log2 fold change in abundance above 20, from ASV transformed count table after variance stabilizing transformation.

2.3.5 Metagenome prediction analysis

For metagenome prediction, the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) pipeline was used directly on the ASVs (Langille et al., 2013). This program uses the 16S rRNA gene marker to predict the metagenomic content of a microbial community through sequence inventories available. The output was analysed using the STAMP software retaining unclassified reads for gene frequencies calculations (Parks et al., 2014), through ANOVAs of effect sizes between groups with Games-Howell post-tests and Bonferroni correction for multiple tests, with corrected p-value<0.001 for differences depending on VIM environment.

2.4 Results

2.4.1 General taxonomy

After reads processing and contaminants filtering, a total read count of 2,353,222 was obtained for gut microbial communities in VIM, with an average counts per sample of 13,524.26 (SD ± 2,934.436) reads. A total number of 16,256 ASVs – or phylotypes – were identified. At the phylum level, Firmicutes largely dominated the fecal samples (Figure 1), representing an average of 72% of all samples (SD ± 0.11), and Bacteroidota (20.5% ± 0.09) followed in greater fluctuating prevalence of Verrucomicrobiota (0.018% ± 0.02), Cyanobacteria (0.016% ± 0.02) and Proteobacteria (0.013% ± 0.04). Free-ranging VIM hosted a greater proportion of

Actinobacteriota and Euryarcheota (Archea kingdom) than captive VIM, while VIM in the *in situ* facility only harbored more Verrucomicrobiota (Figures 1). On the other end, VIM held in *ex situ* facilities had feces enriched with Desulfobacterota, Elusimicrobiota and Cyanobacteria compared to VIM from other locations.

Within the Firmicutes phylum, the *Lachnospiraceae* family was the most abundant in captive VIM (*in situ:* 26% ± 8.4; *ex situ:* 23% ± 6.1), followed by *Oscillospiraceae* and *Ruminococcaceae*, while free-ranging VIM had the *Clostridia UCG-014* as most abundant family (18.2% ± 7.3), followed by *Lachnospiraceae*, *Oscillospiraceae* and *Ruminococcaceae*. Among the Bacteroidota phylum, the *Muribaculaceae* family was the most prevalent in all marmots (9.9% ± 4.6), next to *Rikenellaceae* and *Bacteroidaceae*. On the other end, the Cyanobacteria were largely dominated by the *Gastranaerophiliales* family with great variability between VIM environments (2.1% ± 1.5). In the same way, the most abundant member of the Verrucomicrobiota phylum was the *Akkermansiaceae* family, that was present in low abundance *in ex situ* VIM (0.8% ± 0.7) compared to great variations in *in situ* (2.4% ± 2.3) and free-ranging VIM (1.3% ± 2.3; Figure 2).

For free-ranging VIM captured and translocated at the in situ facility, I observed a reduction in the abundant phyla Firmicutes (Eubacterium coprostanoligenes group, Clostridia UCG-014, Clostridiaceae, Lactobacillaceae, Monoglobaceae, Peptococcaceae, RF39) and Bacteroidota (Muribaculaceae) and an increase for Cyanobacteria (Gastranaerophilales; Figure S1). On the other end, the three VIM that experienced translocation from the in situ to ex situ facilities experienced a loss in Firmicutes (Eubacterium coprostanoligenes group, Clostridia UCG-014, Monoglobaceae, RF39) and Bacteroidota (Marinifilaceae, Enterobacteriaceae), while an enrichment was observed in the phyla Cyanobacteria (Gastranaerophilales), Firmicutes (Clostridia vadinBB60 group, Oscillospiraceae, UCG-010) and Bacteroidota (Muribaculaceae, Rickenellaceae; Figure S1).

2.4.2 Alpha diversity variation

Gut microbial phylotypes richness did not significantly vary according to VIM sex, age, previous location, source of mortality nor outside access, when considering three richness measures on the full dataset (Table 2). For the three indices investigated, the VIM environment had a significant impact on alpha diversity, where VIM held in *ex situ* facilities had all indices higher than other locations (Figure 3). On the other end, a decrease in alpha diversity was reported in observed richness and Faith's PD when VIM were born in the wild, as well as when the dame and sire of this marmot were born in the wild, regardless of its environment at time of sampling (Table 2, S1). Faith's PD index also significantly increased when pups are present in a VIM pair (Table 2). Neither VIM environment or number of days spent in captivity impacted the alpha diversity measures when considering free-ranging VIM

captured and translocated at the *in situ* facility, however, the Faith's PD index gradually increased when VIM held at the *in situ* facility were translocated to an *ex situ* facility (Figure 3).

2.4.3 Beta diversity variation

When considering dissimilarities between GMCs in VIM using weighted Unifrac distances, the model including all the variable tested explained 22.31% of the variation between samples. I observed differences in composition between VIM environments and explained 6.43% of the variation (Adonis: $F=6.3292$; $R^2=0.0643$; $p=0.007$), and centroid dispersion differed between the three environments (Table S2), with greater dispersions to the centroid for the *ex situ* located VIM compared to other locations (permuted $p\text{-value}_{\text{ex situ-in situ}}<0.001$; permuted $p\text{-value}_{\text{ex situ-wild}}<0.001$; Fig. 4A). VIM birthplace also influenced GMCs composition (Adonis: $F=2.2495$; $R^2=0.0571$; $p=0.004$), mostly explained by greater variation in composition from wild-born VIM compared to other factors (permuted $p\text{-value}_{\text{wild-ex situ}}<0.002$; permuted $p\text{-value}_{\text{wild-in\&ex situ}}<0.001$; permuted $p\text{-value}_{\text{wild-in situ\&wild}}<0.05$; Fig. 4B). Parent birthplace also explained 4.5% of the GMCs composition variation (Adonis: $F=1.4801$; $R^2=0.0451$; $p=0.006$), with distinctive compositions from individuals descending from pairs born in *ex situ* facilities and in the wild in Vancouver Island combined (permuted $p\text{-value}_{\text{ex situ\&wild-other}}<0.01$; Fig. 4C), as well as parents coming only from the wild (Table S2). Source of mortality was also a significant variable for GMCs composition (Adonis: $F=1.508$; $R^2=0.0306$; $p=0.005$), with greater dispersions to the centroid for mortality caused by neoplasia or respiratory/bacterial complications (permuted $p\text{-value}_{\text{neoplasia-other}}<0.05$; permuted $p\text{-value}_{\text{respiratory/bacteria-other}}<0.005$; Fig. 4D; Table S2). Finally, Mantel test showed a non-significant negative correlation between GMCs composition and VIM relatedness (Mantel: $r=-0.056$, $p\text{-value}=0.951$).

Neither VIM environment or number of days spent in captivity significantly impacted GMCs composition when free-ranging VIM would be translocated to the *in situ* facility. The turnover component was 0.91, and nestedness of 0.024 between the two locations. However, VIM environment explained 22% of GMCs composition variation during translocation from *in* to *ex situ* facilities (Adonis: $F=2.348$; $R^2=0.2207$; $p=0.011$) with greater intra-individual variation for VIM held in *ex situ* locations (permuted p-value <0.05; Fig. 4E). Moreover, the turnover between the two locations was 0.98 and the nestedness component of 0.006.

2.4.4 Differential abundance

Linear discriminant analysis effect size (LEfSe) method identified the *Clostridium sensu stricto 1* genus as a biomarker for GMCs of free-ranging VIM, while zero taxa were identified for captive VIM at the *in situ* facility (Table S2). On the other end, twelve genera were

significantly enriched in captive VIM in *ex situ* locations across the phyla Firmicutes, Bacteroidota, Cyanobacteria, Spirochaetota and the archeal Thermoplasmatota (Figure 5). Biomarkers were identified according to VIM birthplace for animals born in *ex situ* facilities, including 3 uncultured genera of *Oscillospiraceae* (Firmicutes), *Rickettsiales* and *Rhodospirillales* (Alphaproteobacteria), and for VIM born in captivity (for pairs from both *in* and *ex situ* facilities) that had their GMCs enriched in 34 genera, 50% of which belong to the *Lachnospirales* and *Oscillospirales* orders (Firmicutes; Table S3).

Finally, VIM that died following neoplasia had GMCs significantly enriched in 17 genera from a wide range of phyla of Cyanobacteria (*Gastranaerophilales*), Verrucomicrobiota (genus *Victivallis*), Proteobacteria (*Rhodospirillales, Rickettsiales* orders and *Acinetobacter* genus), Bacteroidota (genera *Alistipes* and *Odoribacter*) and Firmicutes (Christenellaceae, Lachnospiraceae, Oscillospiraceae; Figure S2). On the other end, 8 genera and 19 species were identified as biomarkers of GMCs from VIM that died from respiratory or bacterial related causes, a majority belonging to the Firmicutes phylum, as well as Spirochaetota (*Treponema* genus) and Bacteroidota (*Bacteroides*, *Prevotella* and *Parabacteroides* genera). VIM that died for unknown causes had their GMCs enriched in *Enterorhabdus* genus (Actinobacteriota) and three *Clostridiales* genera (Table S3; Figure S2).

From the two translocation events, no ASV was detected to be differentially abundant for VIM that moved from the wild to the *in situ* facilities, whereas 19 taxa were significantly less abundant at the *in situ* location for the two VIM pairs that experienced a translocation event between *in* to *ex situ* facilities. Among those taxa were three ASVs belonging to the *Bacteroides* genus, one *Christensenellaceae R-7 group*, four from the *Muribaculaceae* genus, two for *Ruminococcus*, one *Alistipes*, two from *UCG-005 Oscillospiraceae*, and five ASVs from the *Lachnospiraceae* family (Table S4).

2.4.5 Metagenome prediction

Finally, I assessed the potential diversity from the bacterial genes present in the available metagenomes of all the samples in our study using the PICRUSt2 algorithm. The average Sequenced Taxon Index (NSTI) is a measure that shows how well a microorganism can be matched to the reference database according to its 16S marker gene. The NSTI did not vary according to VIM environment (Anova: $F=0.416$; $p < 0.1$). As the metagenomes are phylogenetically predicted, the following analysis must be interpreted with caution. Overall abundances of microbial genes related to various KEGG pathways were composed for a majority of genes linked to metabolism, and particularly related to carbohydrates, amino acids and vitamins (Fig. S3). I examined differences in microbial gene abundances by KEGG pathways according to VIM environment. Genes related to metabolism differed between

groups (Eta2=0.111; p-value<0.001), with increased abundance of genes for amino acid and energy metabolism in GMCs of free-ranging VIM, while VIM in *ex situ* facilities had more microbial genes related to glycan and lipid metabolism (Figure 7). Free-ranging VIM were also enriched in gut microbial genes involved in cellular processes (Eta2=0.107; p-value<0.001) and genetic information processing (Eta2=0.130; p-value<0.001).

2.5 Discussion

Our analysis of the VIM microbiota using 16S rRNA amplicon sequencing revealed that it is dominated by Firmicutes (Clostridia), followed by Bacteroidota and Verrucomicrobiota during summer. It is important to acknowledge that seasonal GMCs variation must be important throughout the year as the VIM hibernate for approximatively six months. However, our results are consistent with other hibernating rodents for the active season (reviewed in Carey & Assidy-Porter, 2017). Despite the caution that one must take interpreting the PICRUSt2 results, I observed a great abundance of gut microbial genes involved in carbohydrate and energy metabolism, as seen in other hibernators (Borbón-García et al., 2017), as well as amino acid metabolism, as herbivorous hosts have GMCs specialized to synthesize amino acid building blocks (Muegge et al., 2011).

Examination of GMCs variation through alpha, beta diversity and differential abundance between VIM held in different environments converged toward similar patterns. Increase in diversity and changes in composition were observed in GMCs from VIM held in *ex situ* facilities (zoos) compared to the *in situ* location and the wild. As I expected greater differences between captive and free-ranging VIM, husbandry techniques or geographical location must have a role in the variation of GMCs between captive facilities. While both captive groups of VIM had access to an outside enclosure, it is possible that gut VIM at the MRC (*in situ*) could be colonized by microbes through horizontal transmission from air and substrates that are common to free-ranging VIM (Perofsky et al., 2019). Geographical location can also be reflected in changes in photoperiod between the Vancouver Island and the two zoos (Calgary Zoo and Toronto Zoo). While other studies have detected changes in GMCs according to day length in hibernating Siberian hamsters (Bailey et al., 2010; Ren et al., 2020), further investigation in controlled environments could measure the impact of photoperiod on the marmot GMCs. On the other end, diet variation is known to be a major driver of GMCs in herbivores and rodents, especially when considering both captive and free-ranging animals (Frankel et al., 2019; Van Leeuwen et al., 2020). Differences in diet for VIM between the MRC and the zoos could explain the GMCs variation that I observed, especially based on which raw food the marmots are fed during the active season. At the MRC, the pellet diet (16%) is supplemented with natural vegetation collected directly from the VIM natural habitat, among which *Lupinus* sp is extensively available (McAddie, pers. comment). Supplying food

items directly from the species' natural habitat could therefore provide exposure and specialization of GMCs that are more reflective of free-ranging marmot GMCs (Martinez-Mota et al., 2019). Even when considering the predicted metagenome, I observed similarities in metabolic pathways between *in situ* and wild GMCs, with high proportion of genes involved in amino acid metabolism. In lemurs, increased metabolic pathways for amino acids were observed in foliovore hosts, while frugivore GMCs were enriched in pathways for carbohydrate, glycan, vitamin and cofactor metabolism (Mc Kenney et al., 2017), and I observed similar patterns for the VIM.

At the Toronto Zoo, the pellets are supplemented with other types of raw vegetables (lettuce, kale, broccoli, and cauliflower) and occasionally browse of *Populus* sp and *Malus* sp (Wensvoort pers. comm., 2021). This variation in diet could foster different gut metabolism pathways and thus increase the number of ecological niches within the marmot gut, resulting in higher microbial diversity and inter-individual variation. It is even more striking when we consider the significant results during the translocation event between the MRC and Calgary Zoo compared to the low variation between the natural habitat and the MRC. As found in frugivore lemurs, I observed an increase in the *Treponema* genus and Spirochaetes in *ex situ* VIM (McKenney et al., 2017). The archeal class of *Methanomassiliicoccales* (Thermoplasmatota) was also in greater abundance in *ex situ* GMCs, which is commonly present in the herbivore rumen (Borrel et al., 2020). The prevalence of *Methanomassiliicoccales*, which use H_2 to reduce trimethylamine for methanogenesis, was found to correlate positively with the number of different trimethylamine producing pathways present in bacterial communities (*Lachnospiraceae*; Borrel et al., 2020) and vegetables are known to be a rich dietary source of choline, involved in this pathway.

Many taxa that were significantly more abundant in *ex situ* VIM are also known for butyrate production through carbohydrate fermentation in the gut of herbivorous mammals, such as *Gastranaerophilales* (Di Rienzi et al., 2013), *Oscillospiraceae*, *Lachnospiraceae* and *Butyrivibrio* sp (Amato et al 2013; Meehan & Beiko 2014). Those taxa also varied in abundance according to VIM place of birth and of their parents birthplace, supporting the importance of horizontal transmission and early life exposure from the natural habitat for the VIM (Bokulich et al., 2016; Sonnenburg et al., 2016). However, the fact that the GMCs did not vary according to the VIM's previous location could imply that GMCs variations in *ex situ* VIM could be reversible to some extent. To evaluate how those changes could occur, longitudinal studies during diet manipulation or translocation from zoos to the MRC before reintroduction could be conducted.

I also identified several biomarkers related to causes of mortality in captive VIM and especially related to neoplasia and respiratory/infection causes. While neoplasia enriched

microbial taxa are difficult to link to the condition, routine screenings for these bacteria (*Victivallis* sp, *Flavonifractor plautii*, Table S3) and abundance surveillance could be a non-invasive prophylaxis measure before conducting more invasive tests. Marmots that died from infections or respiratory causes had their GMCs significantly enriched in potential pathogens, even if samples were collected from one year to forty days prior to the VIM death. Among those biomarkers is *Bacteroides massiliensis*, also present in gut biopsies of human suffering ulcerative colitis (Lucke et al., 2006). *Erysipelotrichi* increased abundance in the gastrointestinal tract has also been associated with effects detrimental to human colorectal cancer (Chen et al., 2012) and gastrointestinal inflammation (Schaubeck et al., 2016). Members of the *Izemoplasmatales* order were identified to be active DNA degraders by Wasmund et al. (2020). This taxa was hypothesized to protect the host from invading viruses and pathogens, potentially indicating an over representation of pathogens within the host. Once again, early detection of microbial biomarkers through fecal samples could help veterinarians to limit invasive procedures and mortalities that could be avoided and thus benefit the captive breeding program of the VIM. However, further investigation on seasonal variation and the development of specific markers should be conducted to confirm its utility.

Finally, while speculative, GMCs altered by captivity may affect behaviour relevant to predator avoidance. There is substantial evidence that GMCs affect stress-related behaviours such as exploration (Vuong et al., 2017). For example, germ-free laboratory mice (Heijtz et al., 2011; Neufeld et al., 2011) are more willing to explore open spaces than laboratory mice with normal gut microbiota. Abnormalities in exploratory behaviours were re-established by restoring a normal GMC. Links between exploratory behaviours and GMCs in marmots could be a promising lead to further understand and mitigate excessive predation of VIM in the wild (Jackson et al., 2016).

While this study provides a first in depth look at the GMCs of the Vancouver Island marmot, gut bacteria for this host remains largely unexplored and could harbor taxa with previously unknown metabolic capabilities. I have shown that diet is a critical driver of GMCs in captive marmots, and because of the low overwinter survival in captive-released marmots (Jackson et al., 2016), it is possible that gut microbial metabolic pathways fostered in captivity might not be adequate for hibernation in the natural habitat. For this reason, further omic approaches would allow us to understand the metabolic importance of the marmots' gut microbiota, especially during hibernation between the two types of captive settings. In the light of our results, transition from *ex situ* facilities to *in situ* location before reintroduction in the natural habitat is adequate strategy to foster GMCs specialization. The implementation of management measures such as the stepping-stone approach for the VIM reintroduction increased captive-born VIM survival (Lloyd et al., 2019). Introduction of captive-born marmots in established colonies before translocation in harsher habitats might also be

beneficial for the GMCs angle. However, further research on translocation strategies between captive facilities could also benefit the VIM captive breeding program.

2.6 References

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2.7 Tables and figures

Collection type	Location	Number of individuals (F;M;P)	Number of samples (Min-Max by VIM)
ex situ	Calgary Zoo	2 (0;2;0)	6
	Toronto Zoo	9 (1;0;8)	26
	Sub-total	11 (1;2;8)	32 (2-3)
in situ	TBMWMRC	34 (10;15;9)	98 (2-5)
Vancouver Island	Big Ugly	4	5
	Castlecrag	3	4
	Douglas Peak	1	1
	Flower Ridge	1	1
	Haley Lake	1	1
	Marble Meadow	3	3
	Mount Arrowsmith	7	7
	Mount Moriarty	1	1
	Mount Washington	15	17
	Northwest Bay	3	3
	Steamboat Mountain	1	1
	Sub-total	40 (23;17;0)	44 (1-2)
Total		86 (32;29;17)	176 (1-5)

Table 1. Information on fecal sample collection according to location of VIM individuals, with number of minimal and maximal number of samples collected by VIM (F: female; M: male; P: pair).

Dataset	Response variable	Predictors	Sum Sq	Mean Sq	DenDF	F value	Pr(>F)
Full	Observed richness	*VIM Environment*	*4734*	*2366.98*	*149*	*7.099*	*0.0011*
		Birth place	*6077.4*	*1215.47*	*149*	*3.645*	*0.0038*
		Previous location	1043.3	347.77	*149*	1.043	0.3754
		Sex	412.1	206.07	*149*	0.618	0.5403
		Minimal age	-0.619	1.08	*148*	-0.574	0.5671
		Parent birth location	*8411.9*	*1401.99*	*149*	*4.204*	*0.0006*
		Source of mortatily	1441.6	360.39	*149*	1.081	0.3681
		Presence of pups	391.7	391.74	*149*	1.175	0.2801
		Outside access	16.3	16.31	*149*	0.049	0.8252
	Shannon index	*VIM Environment*	*0.25*	*0.127*	*25*	*3.872*	*0.0339*
		Birth place	0.35	0.071	20.	2.159	0.0987
		Previous location	0.087	0.029	24	0.885	0.4624
		Sex	0.013	0.006	24	0.193	0.8261
		Minimal age	-0.007	0.011	58	-0.601	0.5503
		Parent birth location	*0.587*	*0.097*	*20*	*2.967*	*0.0304*
		Source of mortatily	0.083	0.021	27	0.628	0.6466
		Presence of pups	0.004	0.004	14	0.115	0.7396
		Outside access	0.008	0.008	33	0.255	0.6169
	Faith's PD	*VIM Environment*	*44.3*	*22.162*	*55*	*4.707*	*0.0129*
		Birth place	*87.41*	*17.481*	*46*	*3.713*	*0.0066*
		Previous location	7.42	2.474	53	0.526	0.6665
		Sex	2.94	1.469	52	0.312	0.7332
		Minimal age	0.16	0.133	96	1.237	0.2191
		Parent birth location	*99.42*	*16.57*	*45*	*3.519*	*0.006*
		Source of mortatily	29.4	7.348	58	1.561	0.1967
		Presence of pups	*31.84*	*31.837*	*34*	*6.762*	*0.0137*
		Outside access	1.62	1.624	67	0.345	0.5589
Entrance in captivity	Observed richness	VIM Environment	16.27	16.266	29	0.042	0.8391
		Days spent in captivity	310.1	310.097	15	0.799	0.3849
	Shannon index	VIM Environment	0.021	0.021	34	0.579	0.4519
		Days spent in captivity	0.008	0.008	34	0.231	0.6339
	Faith's PD	VIM Environment	1.33	1.335	34	0.272	0.6052
		Days spent in captivity	2.41	2.413	34	0.492	0.4879
From *in* to *ex situ* facility	Observed richness	VIM Environment	1059.65	1059.65	6	5.81	0.0513
		Days spent in ex situ facility	176.19	176.19	6	0.967	0.3621
	Shannon index	*VIM Environment*	*0.101*	*0.101*	*16*	*8.512*	*0.0246*
		Days spent in ex situ facility	0.005	0.005	16	0.386	0.555
	Faith's PD	VIM Environment	1.33	1.33	6	0.677	0.4419
		Days spent in ex situ facility	*12.204*	*12.204*	*6*	*6.209*	*0.0467*

Table 2. Model results of restricted maximum likelihood fitting linear mixed-effects models for the three datasets investigated according to predictor variables of interest. Italicized values are meeting the significance cutoff of p-value<0.05.

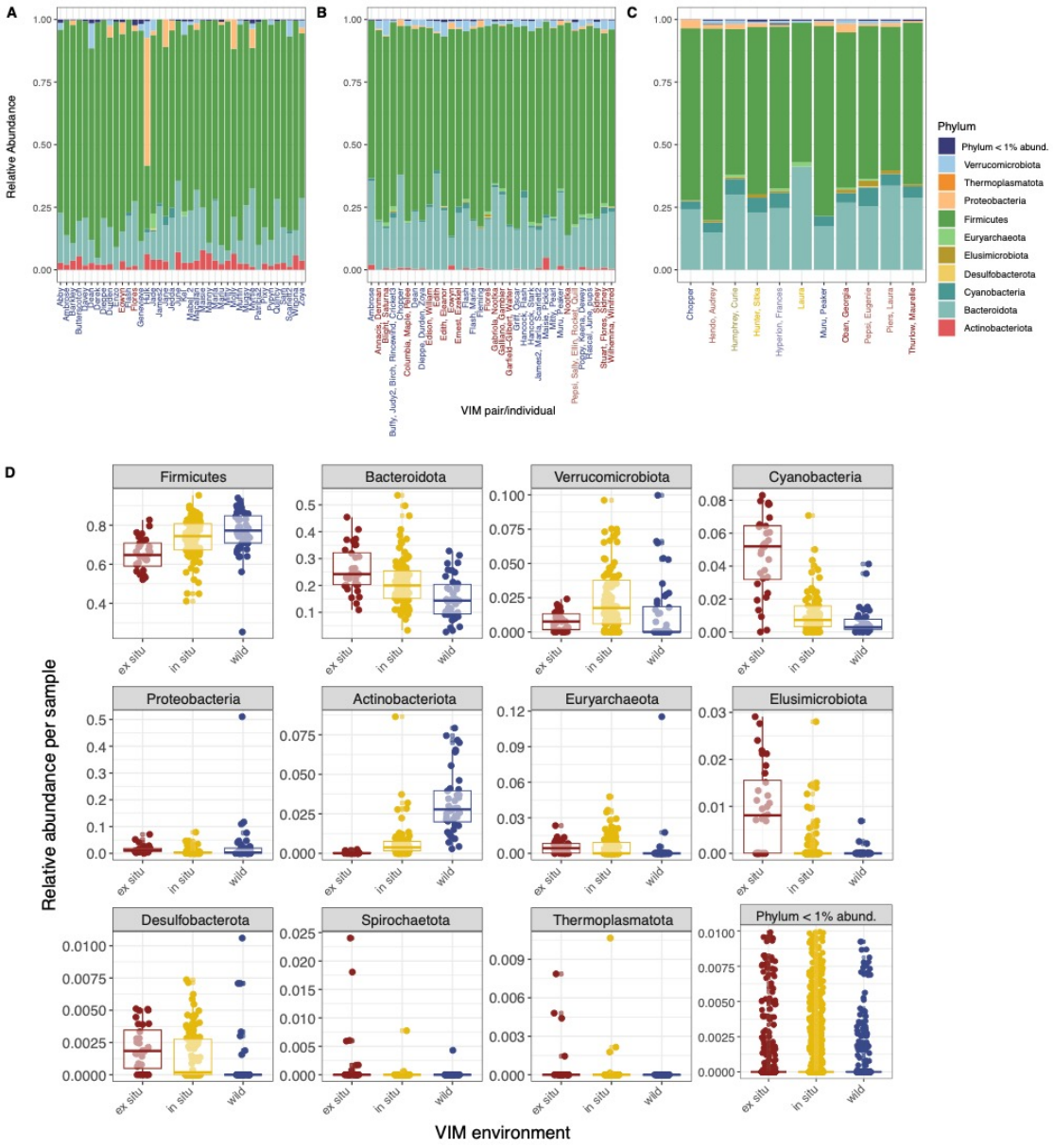

Figure 1. Mean relative abundance of microbial phyla recovered from *M. vancouverensis* fecal samples per pair/individuals located in (A) the natural habitat of Vancouver Island, (B) the *in situ*, and (C) *ex situ* facilities. VIM names are colored by place of birth (dark blue = wild, dark red = *ex situ*, yellow = *in situ*, light red = *in* & *ex situ*, olive green = *ex situ* & wild, light blue = *in situ* & wild). (D) Boxplots of bacterial/archeal phyla relative abundance per sample by VIM environment during sample collection. Absence of boxplot for an environment indicate absence of sequences for the particular phylum.

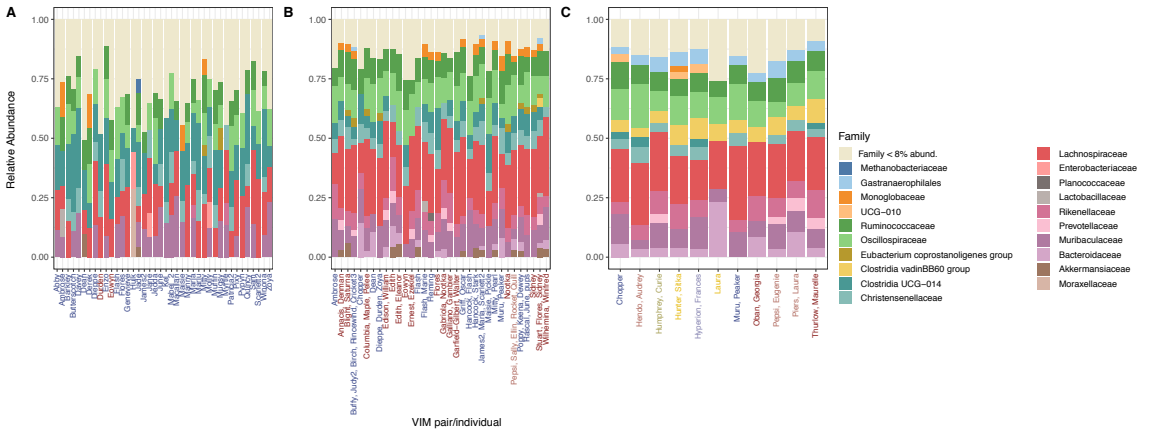

Figure 2. Mean relative abundance of major microbial families recovered from *M. vancouverensis* fecal samples per pair/individuals located in (A) the natural habitat of Vancouver Island, (B) the *in situ*, and (C) *ex situ* facilities (mean total relative abundance above 0.08). VIM names are colored by place of birth (dark blue = wild, dark red = *ex situ*, yellow = *in situ*, light red = *in* & *ex situ*, olive green = *ex situ* & wild, light blue = *in situ* & wild).

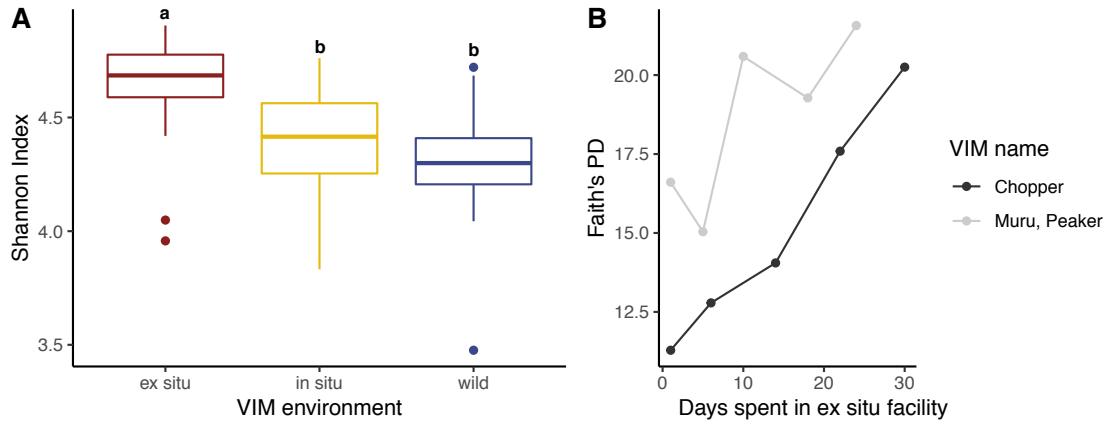

Figure 3. (A) Boxplots of Shannon index variation according to VIM environment for the full dataset. (B) Variation of Faith's PD index according to days spent in captivity for three VIMs (from *in* to *ex situ* facility dataset).

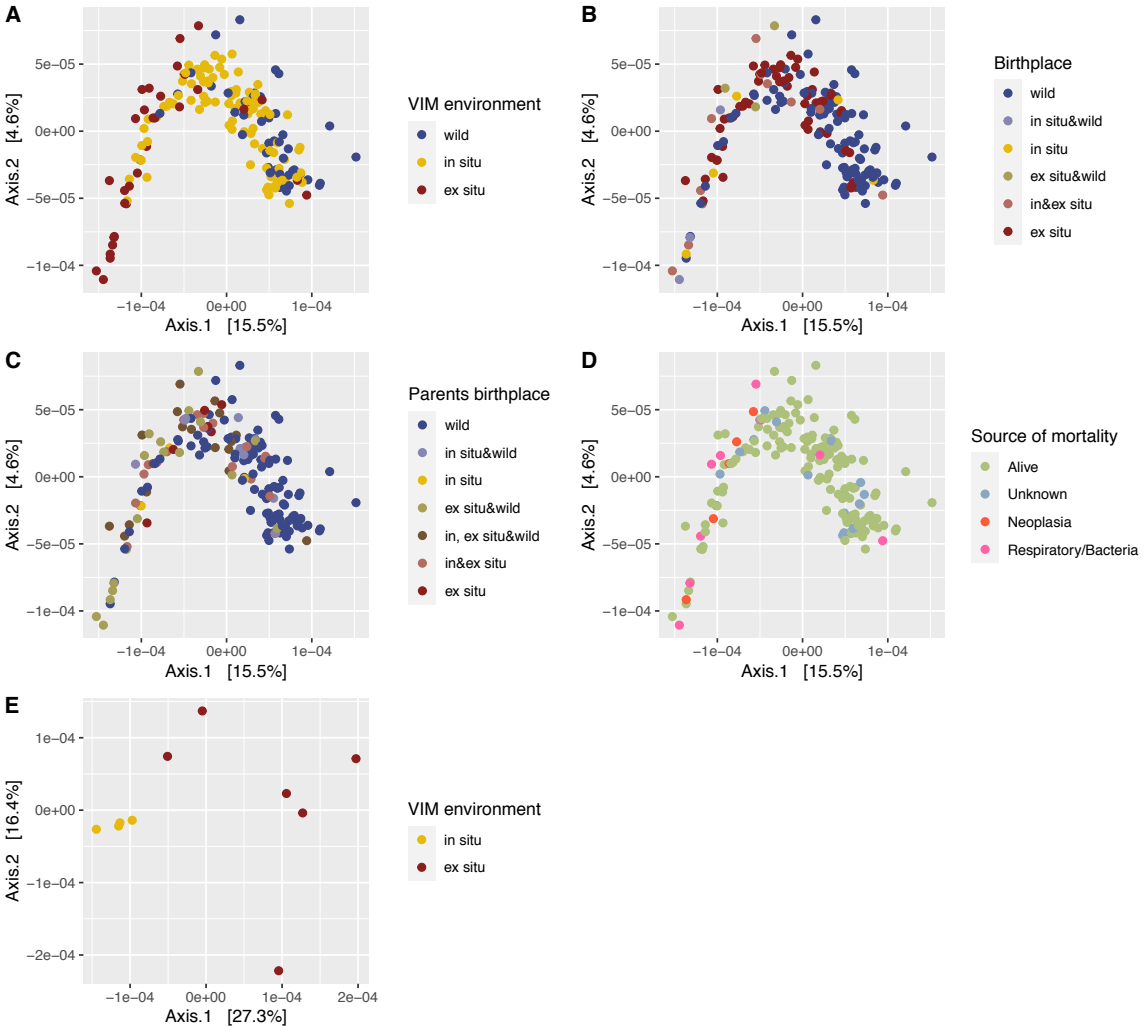

Figure 4. PCoA on weighted Unifrac distances between samples for (A-D) the full dataset and (E) for translocation from *in* to *ex situ* facility according to significant variables from the PERMANOVA models.

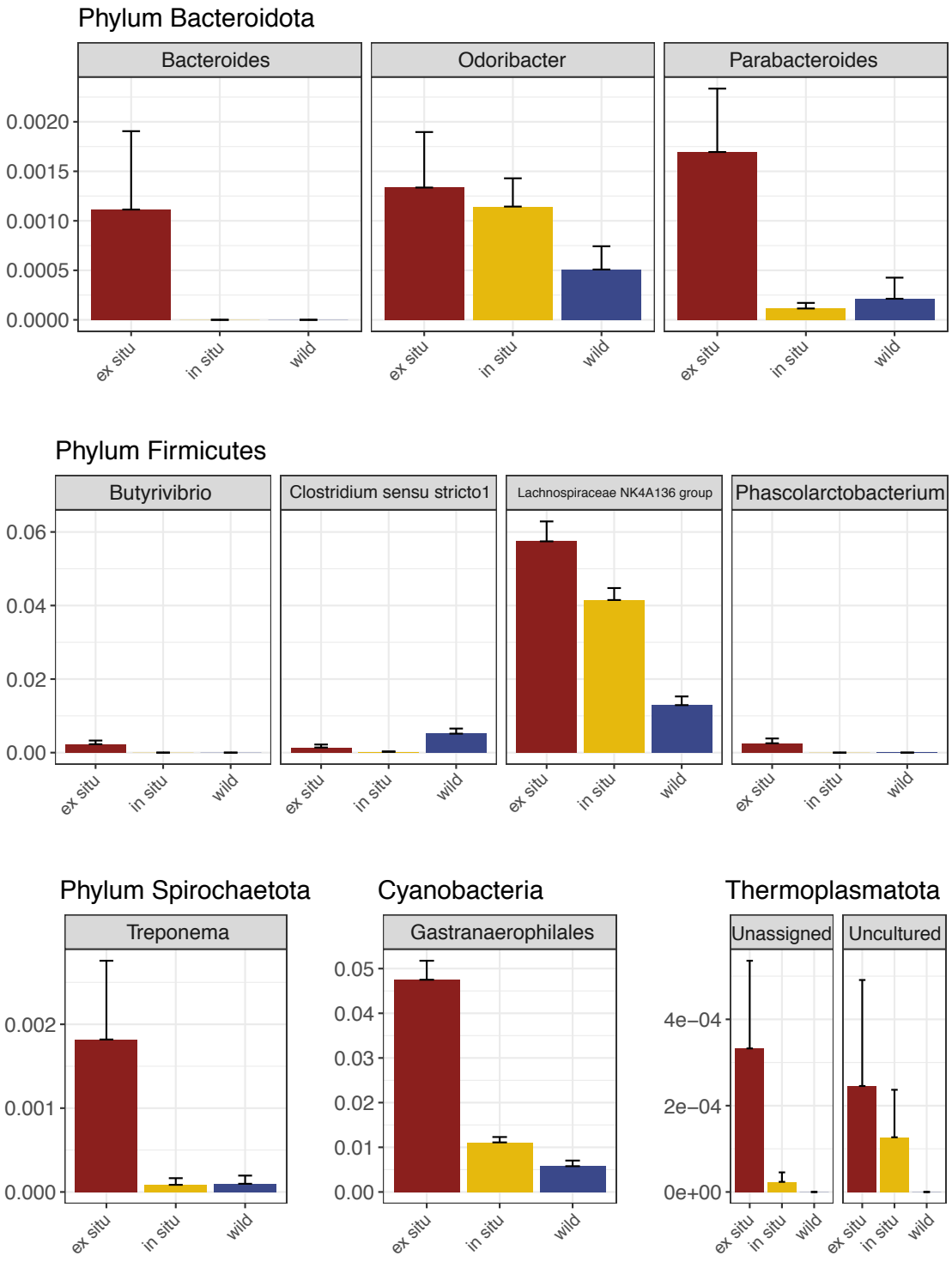

Figure 5. Mean relative abundance (with standard errors) of microbial genera with significant differential abundance recovered from LEfSe analysis depending on VIM environment.

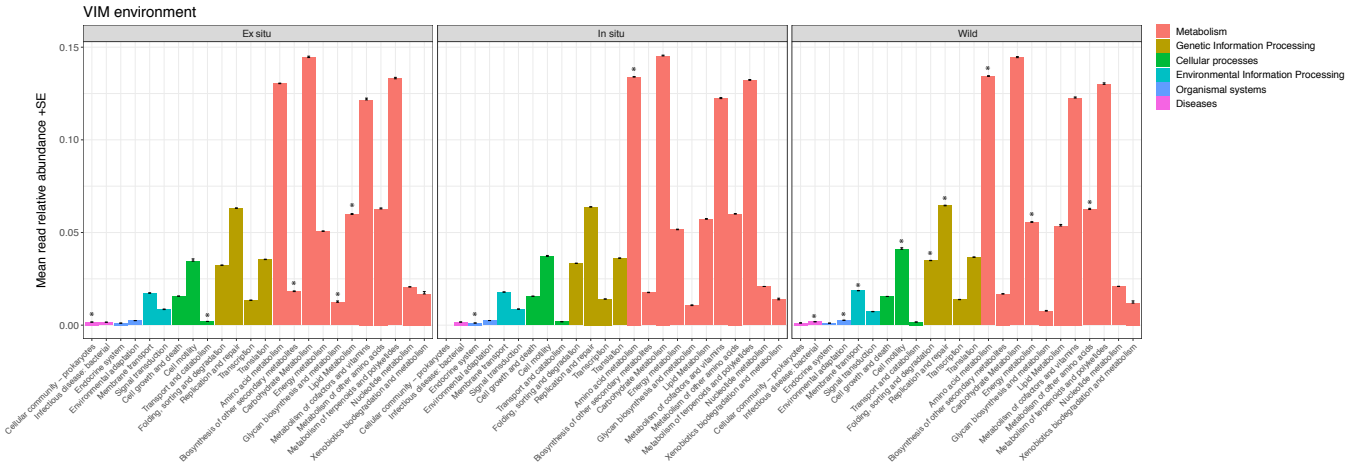

Figure 6. Mean relative abundance (with standard errors) of predicted microbial genes according to KEGG pathways (level 2) by VIM environment. * represents significant post-hoc test for increased abundance for this pathway in the specific VIM group. Only categories with a mean abundance of at least 0.001 are shown.

2.8 Supplementary materials

Dataset	Response variable	Predictors	Level1	Level2	Difference	95%CI	SE	df	t-value	p-value
Full	Observed richness	VIM Environment	ex situ	in situ	39.74	[12.11.67.38]	10.82	26.58	3.67	0.003
			ex situ	wild	41.35	[10.65.72.05]	12.13	30.87	3.41	0.005
		Birthplace	in situ	wild	-55.40	[-106.24.-4.55]	16.50	50.58	-3.36	0.018
		Parent birth location	ex situ	wild	108.11	[13.06.203.16]	29.05	35.47	3.72	0.011
			ex situ & wild	wild	101.82	[15.38.188.26]	26.42	35.49	3.85	0.008
			in & ex situ	wild	101.88	[11.03.192.74]	27.74	35.03	3.67	0.013
			in. ex situ & wild	wild	97.24	[-3.17.197.66]	30.55	33.51	3.18	0.045
			in situ	wild	111.90	[12.38.211.42]	30.46	36.16	3.67	0.012
	Shannon index	VIM Environment	ex situ	in situ	0.31	[0.02.0.60]	0.11	27.23	2.71	0.030
			ex situ	wild	0.34	[0.01.0.66]	0.13	31.50	2.62	0.035
	Faith's PD	VIM Environment	ex situ	in situ	3.90	[0.39.7.41]	1.38	27.25	2.83	0.023
			ex situ	wild	4.63	[0.74.8.51]	1.54	31.52	3.01	0.014
		Birthplace	ex situ	wild	-11.06	[-21.95.-0.17]	3.46	35.45	-3.20	0.032
			in & ex situ	wild	-13.06	[-26.44.0.32]	4.21	30.81	-3.10	0.043
		Parent birth location	ex situ	wild	14.54	[2.57.26.51]	3.66	36.08	3.97	0.006
			ex situ & wild	wild	13.80	[2.91.24.70]	3.34	36.28	4.14	0.003
			in & ex situ	wild	13.87	[2.42.25.32]	3.50	35.77	3.96	0.006
			in. ex situ & wild	wild	14.43	[1.77.27.09]	3.86	34.46	3.74	0.011
			in situ	wild	15.57	[3.03.28.11]	3.84	36.76	4.05	0.004
			in situ & wild	wild	13.67	[1.79.25.55]	3.63	35.37	3.77	0.010
		Presence of pups	no	yes	-2.74	[-4.99.-0.50]	1.08	2	-2.53	0.019
From *in* to *ex situ* facility	Shannon index	VIM Environment	ex situ	in situ	0.38	[0.04.0.71]	0.14	2	2.68	0.034
	Faith's PD	Days spent in ex situ facility			0.217		0.087	6.049	2.492	0.0467

Table S1. Contrast value of restricted maximum likelihood fitting linear mixed-effects models for the three datasets investigated according to significant predictor variables of interest. Only values meeting the significance cutoff of p-value<0.05 are represented.

Dataset	Variable	Level1	Level2	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Full	VIM environment	in situ	wild	1.7830e-07	1.7830e-07	3.4612	0.02413	0.000999
		ex situ	in situ	4.2200e-07	4.2198e-07	5.9436	0.04437	0.000999
		ex situ	wild	6.8260e-07	6.8255e-07	8.5014	0.10305	0.000999
	Birthplace	in situ & wild	wild	2.8840e-07	2.8838e-07	5.2999	0.05131	0.000999
		in situ	wild	1.3610e-07	1.3614e-07	2.4959	0.02435	0.008991
		ex situ & wild	wild	1.5940e-07	1.5940e-07	2.9743	0.02946	0.003996
		in, ex situ & wild	wild	2.2170e-07	2.2171e-07	3.7593	0.03394	0.000999
		ex situ	wild	3.0780e-07	3.0781e-07	5.2641	0.03412	0.000999
		ex situ	in situ & wild	2.1840e-07	2.1844e-07	3.0293	0.0522	0.000999
	Parent birth location	in situ & wild	wild	8.7700e-08	8.7733e-08	1.6333	0.01503	0.03297
		in situ & wild	ex situ & wild	1.6218e-07	1.6218e-07	1.6808	0.06072	0.01199
		in, ex situ & wild	wild	2.6610e-07	2.6609e-07	4.5683	0.03758	0.000999
		in & ex situ	wild	1.6590e-07	1.6592e-07	3.0132	0.02553	0.000999
		ex situ	wild	1.2480e-07	1.2476e-07	2.3605	0.0224	0.005994
		ex situ & wild	wild	4.1560e-07	4.1558e-07	6.791	0.05576	0.000999
		in & ex situ	ex situ & wild	1.3350e-07	1.3353e-07	1.4677	0.04138	0.02897
	Mortality	alive	respiratory/bacteria	2.0240e-07	2.0240e-07	2.9957	0.02011	0.000999
		alive	neoplasia	2.2240e-07	2.2242e-07	3.3906	0.02316	0.000999
		unknown	respiratory/bacteria	1.5425e-07	1.5425e-07	2.0245	0.07224	0.003996
		unknown	neoplasia	1.9131e-07	1.9131e-07	2.9391	0.11331	0.001998

Table S2. Pairwise PERMANOVA models for significant variables in overall PERMANOVA model. Only values meeting the significance cutoff of p-value<0.05 are represented.

Taxonomy	Variable	Level	log lda	p-value
d__Archaea.p__Thermoplasmatota	VIM_environment	exsitu	4,140	1,15E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata	VIM_environment	exsitu	4,091	1,15E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales	VIM_environment	exsitu	4,134	1,15E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales.f__Methanomethylophilaceae	VIM_environment	exsitu	4,114	1,15E-02
d__Bacteria.p__Bacteroidota	VIM_environment	exsitu	4,681	4,08E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia	VIM_environment	exsitu	4,681	4,08E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales	VIM_environment	exsitu	4,682	6,61E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__Bacteroides_intestinalis	VIM_environment	exsitu	3,871	1,15E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Marinifilaceae	VIM_environment	exsitu	3,741	4,05E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Marinifilaceae.g__Odoribacter	VIM_environment	exsitu	3,737	1,19E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Tannerellaceae	VIM_environment	exsitu	4,180	1,15E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Tannerellaceae.g__Parabacteroides	VIM_environment	exsitu	4,198	1,15E-02
d__Bacteria.p__Cyanobacteria	VIM_environment	exsitu	4,540	1,70E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia	VIM_environment	exsitu	4,529	1,70E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales	VIM_environment	exsitu	4,528	1,70E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales	VIM_environment	exsitu	4,546	1,70E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales	VIM_environment	exsitu	4,545	1,70E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004	VIM_environment	exsitu	3,640	1,20E-04

d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Butyrivibrio	VIM_environment	exsitu	3,758	1,15E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group	VIM_environment	exsitu	4,432	1,19E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales	VIM_environment	exsitu	4,958	2,65E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae	VIM_environment	exsitu	4,760	1,38E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Ruminococcus.s__Ruminococcus_sp_	VIM_environment	exsitu	4,456	1,19E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010	VIM_environment	exsitu	4,014	6,14E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010	VIM_environment	exsitu	4,004	6,14E-03
d__Bacteria.p__Firmicutes.c__Negativicutes.o__Acidaminococcales.f__Acidaminococcaceae.g__Phascolarctobacterium.s__uncultured_organism	VIM_environment	exsitu	4,090	1,15E-02
d__Bacteria.p__Spirochaetota	VIM_environment	exsitu	4,417	1,15E-02
d__Bacteria.p__Spirochaetota.c__Spirochaetia	VIM_environment	exsitu	4,318	1,15E-02
d__Bacteria.p__Spirochaetota.c__Spirochaetia.o__Spirochaetales	VIM_environment	exsitu	4,345	1,15E-02
d__Bacteria.p__Spirochaetota.c__Spirochaetia.o__Spirochaetales.f__Spirochaetaceae	VIM_environment	exsitu	4,372	1,15E-02
d__Bacteria.p__Spirochaetota.c__Spirochaetia.o__Spirochaetales.f__Spirochaetaceae.g__Treponema	VIM_environment	exsitu	4,356	1,15E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales	VIM_environment	wild	4,276	4,03E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae	VIM_environment	wild	4,289	4,03E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__Clostridium_sensu_stricto_1	VIM_environment	wild	4,309	4,03E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__uncultured	birthplace	ex_situ	4,544	3,02E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rhodospirillales	birthplace	ex_situ	4,127	2,90E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rhodospirillales.f__uncultured	birthplace	ex_situ	4,073	2,90E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rhodospirillales.f__uncultured.g__uncultured	birthplace	ex_situ	4,102	2,90E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales	birthplace	ex_situ	4,037	4,64E-03
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__uncultured	birthplace	ex_situ	4,047	4,64E-03
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__uncultured.g__uncultured	birthplace	ex_situ	4,033	4,64E-03
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales.f__Methanomethylophilaceae.g__uncultured	birthplace	in_ex_situ	4,300	1,91E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales.f__Methanomethylophilaceae.g__uncultured.s__gut_metagenome	birthplace	in_ex_situ	4,334	1,91E-02
d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Actinomycetales.f__Actinomycetaceae.g__Actinomyces.s__Actinomyces_succiniciruminis	birthplace	in_ex_situ	3,572	1,91E-02
d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Micrococcales.f__Brevibacteriaceae	birthplace	in_ex_situ	4,481	1,91E-02
d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Micrococcales.f__Brevibacteriaceae.g__Brevibacterium	birthplace	in_ex_situ	4,492	1,91E-02
d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Micrococcales.f__Brevibacteriaceae.g__Brevibacterium.s__Brevibacterium_senegalense	birthplace	in_ex_situ	4,496	1,91E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__Bacteroides_massiliensis	birthplace	in_ex_situ	4,213	1,91E-02

d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__Bacteroides_oleiciplenus	birthplace	in_ex_situ	4,418	1,91E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Prevotella.s__Trichuris_trichiura	birthplace	in_ex_situ	3,823	1,91E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Tannerellaceae	birthplace	in_ex_situ	4,298	3,91E-04
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Tannerellaceae.g__Parabacteroides	birthplace	in_ex_situ	4,285	3,91E-04
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Tannerellaceae.g__Parabacteroides.s__Parabacteroides_johnsonii	birthplace	in_ex_situ	4,235	1,91E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales.s__gut_metagenome	birthplace	in_ex_situ	4,498	1,91E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales.s__uncultured_rumen	birthplace	in_ex_situ	4,454	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004	birthplace	in_ex_situ	3,988	1,43E-05
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004.	birthplace	in_ex_situ	3,935	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004.s__bacterium_YGD2005	birthplace	in_ex_situ	3,837	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004.s__uncultured_bacterium	birthplace	in_ex_situ	3,857	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Faecalitalea.s__uncultured_bacterium	birthplace	in_ex_situ	3,859	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Izemoplasmatales.f__Izemoplasmatales.g__Izemoplasmatales.s__gut_metagenome	birthplace	in_ex_situ	4,085	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Lactococcus	birthplace	in_ex_situ	3,710	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Lactococcus.s__Lactococcus_lactis	birthplace	in_ex_situ	3,815	1,91E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__RF39.f__RF39.g__RF39.s__unidentified_rumen	birthplace	in_ex_situ	3,642	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Christensenellales.f__Christensenellaceae.g__Christensenellaceae_R_7_group.s__Christensenella_sp_	birthplace	in_ex_situ	3,869	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Christensenellales.f__Christensenellaceae.g__Christensenellaceae_R_7_group.s__gut_metagenome	birthplace	in_ex_situ	4,092	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridia_vadinBB60_group.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group.s__uncultured_bacterium	birthplace	in_ex_situ	3,982	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridia_vadinBB60_group.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group.s__uncultured_prokaryote	birthplace	in_ex_situ	3,744	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g___Eubacterium__ventriosum_group.s__uncultured_Eubacterium	birthplace	in_ex_situ	4,420	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Butyrivibrio.s__Butyrivibrio_crossotus	birthplace	in_ex_situ	4,084	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Frisingicoccus	birthplace	in_ex_situ	4,244	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Frisingicoccus.s__uncultured_organism	birthplace	in_ex_situ	4,213	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnoclostridium.s__uncultured_Firmicutes	birthplace	in_ex_situ	4,240	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group.	birthplace	in_ex_situ	4,599	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG_010.	birthplace	in_ex_situ	4,332	1,91E-02

d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__uncultured.s__Clostridiaceae_bacterium	birthplace	in_ex_situ	4,310	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f___Eubacterium__coprostanoligenes_group.g___Eubacterium__coprostanoligenes_group.s__gut_metagenome	birthplace	in_ex_situ	4,521	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__Colidextribacter.s__bacterium_enrichment	birthplace	in_ex_situ	4,231	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__Colidextribacter.s__uncultured_Clostridia	birthplace	in_ex_situ	3,968	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_005.s__uncultured_rumen	birthplace	in_ex_situ	4,359	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_007	birthplace	in_ex_situ	4,087	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_007.s__uncultured_rumen	birthplace	in_ex_situ	4,084	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g___Eubacterium__siraeum_group.s__gut_metagenome	birthplace	in_ex_situ	4,119	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__CAG_352.s__uncultured_Ruminococcus	birthplace	in_ex_situ	3,675	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Candidatus_Soleaferrea.s__Ruminococcaceae_bacterium	birthplace	in_ex_situ	3,747	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Paludicola	birthplace	in_ex_situ	3,982	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Paludicola.s__uncultured_bacterium	birthplace	in_ex_situ	3,987	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010.s__uncultured_bacterium	birthplace	in_ex_situ	4,027	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010.s__uncultured_organism	birthplace	in_ex_situ	3,751	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales_Tissierellales.f__Anaerovoracaceae.g___Eubacterium__nodatum_group.s__uncultured_organism	birthplace	in_ex_situ	4,213	1,91E-02
d__Bacteria.p__Firmicutes.c__Negativicutes.o__Veillonellales_Selenomonadales.f__Veillonellaceae.g__Veillonella.	birthplace	in_ex_situ	3,737	1,91E-02
d__Bacteria.p__Spirochaetota.c__Spirochaetia.o__Spirochaetales.f__Spirochaetaceae.g__Treponema.s__uncultured_bacterium	birthplace	in_ex_situ	4,502	1,91E-02
d__Bacteria.p__Verrucomicrobiota.c__Verrucomicrobiae.o__Opitutales	birthplace	in_ex_situ	3,632	1,91E-02
d__Bacteria.p__Verrucomicrobiota.c__Verrucomicrobiae.o__Opitutales.f__Puniceicoccaceae	birthplace	in_ex_situ	3,788	1,91E-02
d__Bacteria.p__Verrucomicrobiota.c__Verrucomicrobiae.o__Opitutales.f__Puniceicoccaceae.g__uncultured	birthplace	in_ex_situ	3,768	1,91E-02
d__Bacteria.p__Verrucomicrobiota.c__Verrucomicrobiae.o__Opitutales.f__Puniceicoccaceae.g__uncultured.s__metagenome	birthplace	in_ex_situ	3,791	1,91E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae	parent_birthplace	in_situ_wild	5,199	1,65E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Staphylococcales	parent_birthplace	ex_situ_wild	4,312	4,65E-02
d__Bacteria.p__Firmicutes.c__Bacilli.o__Staphylococcales.f__Staphylococcaceae	parent_birthplace	ex_situ_wild	4,266	4,65E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales	cause_death	alive	3,721	1,11E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__uncultured	cause_death	alive	3,751	1,11E-02
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__uncultured.g__uncultured	cause_death	alive	3,729	1,11E-02
d__Archaea.p__Thermoplasmatota	cause_death	Neoplasia	4,311	1,11E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata	cause_death	Neoplasia	4,263	1,11E-02

d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales	cause_death	Neoplasia	4,318	1,11E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales.f__Methanomethylophilaceae	cause_death	Neoplasia	4,342	1,11E-02
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales.f__Methanomethylophilaceae.g__Candidatus_Methanomethylophilus	cause_death	Neoplasia	4,307	1,25E-05
d__Archaea.p__Thermoplasmatota.c__Thermoplasmata.o__Methanomassiliicoccales.f__Methanomethylophilaceae.g__Candidatus_Methanomethylophilus.s__Candidatus_Methanomethylophilus	cause_death	Neoplasia	4,256	1,25E-05
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Marinifilaceae.g__Odoribacter.s__Odoribacter_splanchnicus	cause_death	Neoplasia	3,873	1,25E-05
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__Alistipes	cause_death	Neoplasia	4,339	3,33E-02
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__Alistipes.s__Alistipes_obesi	cause_death	Neoplasia	4,099	1,25E-05
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__Alistipes.s__Alistipes_sp_	cause_death	Neoplasia	4,203	1,25E-05
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales.s__uncultured_cyanobacterium	cause_death	Neoplasia	3,893	1,25E-05
d__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Bacillaceae	cause_death	Neoplasia	4,384	1,33E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Bacillaceae.g__Bacillus	cause_death	Neoplasia	4,421	1,33E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Bacillaceae.g__Bacillus.	cause_death	Neoplasia	4,393	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Christensenellales.f__Christensenellaceae.g__Christensenellaceae_R_7_group.s__human_gut	cause_death	Neoplasia	4,727	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridia_vadinBB60_group.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group.	cause_death	Neoplasia	4,811	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridia_vadinBB60_group.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group.s__unidentified	cause_death	Neoplasia	4,507	9,67E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__Clostridium_sensu_stricto_1.s__human_gut	cause_death	Neoplasia	3,753	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Butyrivibrio	cause_death	Neoplasia	4,229	1,71E-04
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Butyrivibrio.	cause_death	Neoplasia	4,287	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group	cause_death	Neoplasia	5,082	1,25E-08
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group.s__gut_metagenome	cause_death	Neoplasia	4,969	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group.s__uncultured_bacterium	cause_death	Neoplasia	4,479	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__Intestinimonas.s__Flavonifractor_plautii	cause_death	Neoplasia	4,598	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__NK4A214_group.	cause_death	Neoplasia	4,641	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_005.s__human_gut	cause_death	Neoplasia	3,574	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_005.s__uncultured_Sporobacter	cause_death	Neoplasia	4,565	1,25E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__uncultured.s__uncultured_bacterium	cause_death	Neoplasia	4,259	1,25E-05
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rhodospirillales.f__uncultured.g__uncultured.s__gut_metagenome	cause_death	Neoplasia	4,096	1,25E-05

d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__uncultured.g__uncultured.s__uncultured_Alphaproteobacteria	cause_death	Neoplasia	3,545	1,25E-05
d__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Moraxellaceae.g__Acinetobacter.	cause_death	Neoplasia	4,047	1,25E-05
d__Bacteria.p__Verrucomicrobiota.c__Lentisphaeria	cause_death	Neoplasia	4,002	1,25E-05
d__Bacteria.p__Verrucomicrobiota.c__Lentisphaeria.o__Victivallales	cause_death	Neoplasia	4,026	1,25E-05
d__Bacteria.p__Verrucomicrobiota.c__Lentisphaeria.o__Victivallales.f__Victivallaceae	cause_death	Neoplasia	4,026	1,25E-05
d__Bacteria.p__Verrucomicrobiota.c__Lentisphaeria.o__Victivallales.f__Victivallaceae.g__Victivallis	cause_death	Neoplasia	3,988	1,25E-05
d__Bacteria.p__Verrucomicrobiota.c__Lentisphaeria.o__Victivallales.f__Victivallaceae.g__Victivallis.s__uncultured_bacterium	cause_death	Neoplasia	3,989	1,25E-05
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__Bacteroides_massiliensis	cause_death	respiratory_bacteria	4,295	1,06E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__Bacteroides_oleiciplenus	cause_death	respiratory_bacteria	4,479	1,06E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Prevotella.s__Trichuris_trichiura	cause_death	respiratory_bacteria	3,808	1,06E-03
d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Tannerellaceae.g__Parabacteroides.s__Parabacteroides_johnsonii	cause_death	respiratory_bacteria	4,508	1,06E-03
d__Bacteria.p__Cyanobacteria	cause_death	respiratory_bacteria	4,938	2,04E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia	cause_death	respiratory_bacteria	4,929	2,04E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales	cause_death	respiratory_bacteria	4,911	2,04E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales	cause_death	respiratory_bacteria	4,939	2,04E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales	cause_death	respiratory_bacteria	4,950	2,04E-02
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales.s__gut_metagenome	cause_death	respiratory_bacteria	4,828	1,06E-03
d__Bacteria.p__Cyanobacteria.c__Vampirivibrionia.o__Gastranaerophilales.f__Gastranaerophilales.g__Gastranaerophilales.s__uncultured_rumen	cause_death	respiratory_bacteria	4,436	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__Erysipelotrichaceae_UCG_003	cause_death	respiratory_bacteria	3,734	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__Erysipelotrichaceae_UCG_003.s__uncultured_bacterium	cause_death	respiratory_bacteria	3,872	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004	cause_death	respiratory_bacteria	3,962	2,26E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004.	cause_death	respiratory_bacteria	3,947	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelatoclostridiaceae.g__UCG_004.s__uncultured_bacterium	cause_death	respiratory_bacteria	3,813	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Izemoplasmatales.f__Izemoplasmatales.g__Izemoplasmatales.s__gut_metagenome	cause_death	respiratory_bacteria	3,975	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Lactococcus	cause_death	respiratory_bacteria	3,679	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Lactococcus.s__Lactococcus_lactis	cause_death	respiratory_bacteria	3,569	1,06E-03
d__Bacteria.p__Firmicutes.c__Bacilli.o__RF39.f__RF39.g__RF39.s__unidentified_rumen	cause_death	respiratory_bacteria	3,692	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g___Eubacterium__ventriosum_group.s__uncultured_Eubacterium	cause_death	respiratory_bacteria	4,335	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Butyrivibrio.s__Butyrivibrio_crossotus	cause_death	respiratory_bacteria	4,234	1,06E-03

d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group.	cause_death	respiratory_bacteria	4,772	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f___Eubacterium__coprostanoligenes_group.g___Eubacterium__coprostanoligenes_group.s__gut_metagenome	cause_death	respiratory_bacteria	4,425	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__Colidextribacter.s__uncultured_Clostridia	cause_death	respiratory_bacteria	4,108	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_005	cause_death	respiratory_bacteria	4,871	6,98E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_005.s__uncultured_rumen	cause_death	respiratory_bacteria	4,352	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG_005.s__unidentified	cause_death	respiratory_bacteria	4,812	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g___Eubacterium__siraeum_group.s__gut_metagenome	cause_death	respiratory_bacteria	4,192	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010	cause_death	respiratory_bacteria	4,495	2,45E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010	cause_death	respiratory_bacteria	4,487	2,45E-05
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010.	cause_death	respiratory_bacteria	4,198	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010.s__uncultured_bacterium	cause_death	respiratory_bacteria	4,150	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__UCG_010.g__UCG_010.s__uncultured_Ruminococcaceae	cause_death	respiratory_bacteria	3,893	1,06E-03
d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptococcales.f__Peptococcaceae.g__uncultured.s__unidentified	cause_death	respiratory_bacteria	4,142	1,06E-03
d__Bacteria.p__Spirochaetota.c__Spirochaetia.o__Spirochaetales.f__Spirochaetaceae.g__Treponema.	cause_death	respiratory_bacteria	4,611	1,06E-03
d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Enterorhabdus	cause_death	unknown	4,253	2,45E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g___Eubacterium__xylanophilum_group	cause_death	unknown	4,515	2,30E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG_006	cause_death	unknown	4,102	2,30E-02
d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__uncultured	cause_death	unknown	4,639	3,96E-02

Table S3. Additional file: results output from LEfSe for group comparisons of VIM environment, birthplace, parent birthplace and cause of death.

ASV	baseMean	log2FoldChange	lfcSE	padj	Phylum	Class	Order	Family	Genus	Species	abundant in group
0ec007c83d3d227b9d506f643e04c280	23.44310	-22.17062	3.117943	2.742983e-12	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides		ex situ
1d17014036660 28eed2503c4a1d6e531	29.11754	-22.46376	3.117671	2.058644e-12	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides		ex situ
ad46616c52b098e3921ae88a93cec7c0	40.50619	-22.91823	3.117354	1.612011e-12	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides		ex situ
14d63b5a7c53c80bae1206b7b92fd3e8	22.87113	-22.07773	3.117978	3.204135e-12	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae	uncultured bacterium	ex situ
22cc44da9c0c148d5f19ce73f7a708f3	23.53555	-22.17394	3.117938	2.742983e-12	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae	uncultured bacterium	ex situ
808e07c303ebf66fa30b72096301a191	28.19746	-22.42611	3.117707	2.058644e-12	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae	uncultured bacterium	ex situ
26a2669dd840c4d7f91741e4e5f2ce5f	42.26704	-22.97483	3.117320	1.612011e-12	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculaceae	uncultured bacterium	ex situ
64dcf9d7f91ac09f8ab695a1f6cef694	26.60701	-22.34793	3.117777	2.226022e-12	Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes		ex situ
9f16e312c568ce1fda24cb17dd5d8d1a	34.11284	-22.67978	3.117506	1.645799e-12	Firmicutes	Clostridia	Christensenellales	Christensenellaceae	Christensenellaceae R-7 group	uncultured bacterium	ex situ
818be1b5e9b78a765312374aadb4a141	36.04996	-22.75787	3.117454	1.612011e-12	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	uncultured	uncultured bacterium	ex situ
f12a07fbdc3822784fe53865be2b3768	36.53479	-22.77535	3.117442	1.612011e-12	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	uncultured	uncultured bacterium	ex situ
7f524b4778b55c2dd641caecea38ce9b	25.56027	-21.34159	3.117828	1.614259e-11	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	uncultured		ex situ
bec7aa23e97346f6434431df25a999a3	39.84167	-22.88706	3.117367	1.612011e-12	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	uncultured		ex situ
a40089fec24b76ca1a732bb7122b7a98	53.58536	-23.27400	3.117156	1.612011e-12	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	uncultured		ex situ
decf81d185ba8d9b5ff2ef87cd44463c	16.03740	-20.74575	3.118591	5.771532e-11	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae			ex situ
d7c1de8b3f6704246d1aa39681895cb5	24.02348	-22.20992	3.117909	2.742983e-12	Firmicutes	Clostridia	Lachnospirales	Oscillospiraceae	UCG-005	uncultured prokaryote	ex situ
b1c4bb1efa6e0bad79db8e9a27acc213	29.19145	-22.45515	3.117668	2.058644e-12	Firmicutes	Clostridia	Lachnospirales	Oscillospiraceae	UCG-005	uncultured prokaryote	ex situ
ffec34c3eb86bca655247b391b0a4c7b	35.66768	-22.74431	3.117464	1.612011e-12	Firmicutes	Clostridia	Lachnospirales	Ruminococcaceae	Ruminococcus	Ruminococcus sp.	ex situ
11deb688ad8f14bcb5d7f31227bfad9a	28.14813	-22.41490	3.117709	2.058644e-12	Firmicutes	Clostridia	Lachnospirales	Ruminococcaceae	Ruminococcus		ex situ

Table S4. Differentially abundant taxa from DESeq2 analysis for the translocation event between *in* and *ex situ* facilities at the significance cut-off value p-value<0.001.

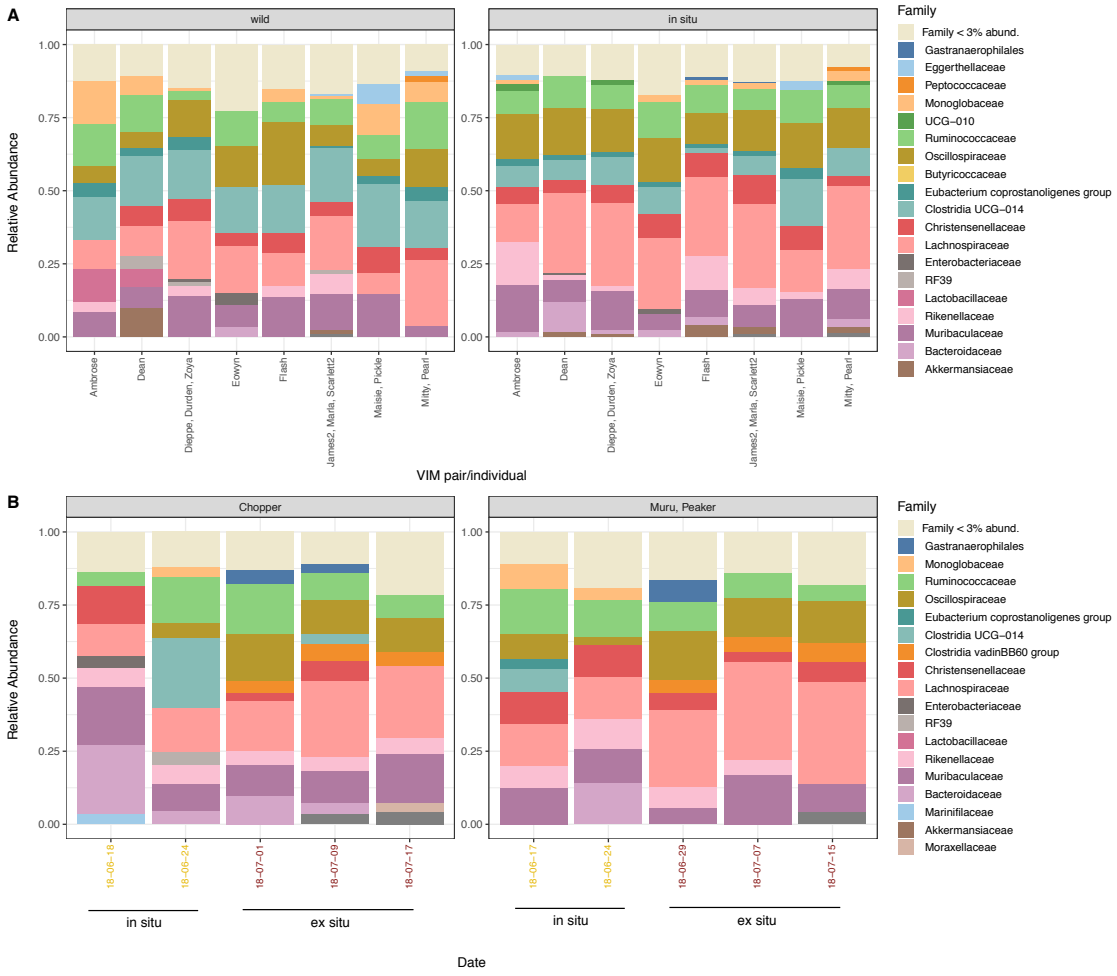

Figure S1. Relative abundance of major microbial families recovered from *M. vancouverensis* fecal samples per pair/individuals located in (A) the natural habitat of Vancouver Island and the *in situ* facility the following day and (B) from the *in situ* to *ex situ* facility according to sampling date for the three VIMs (mean total relative abundance above 0.03).

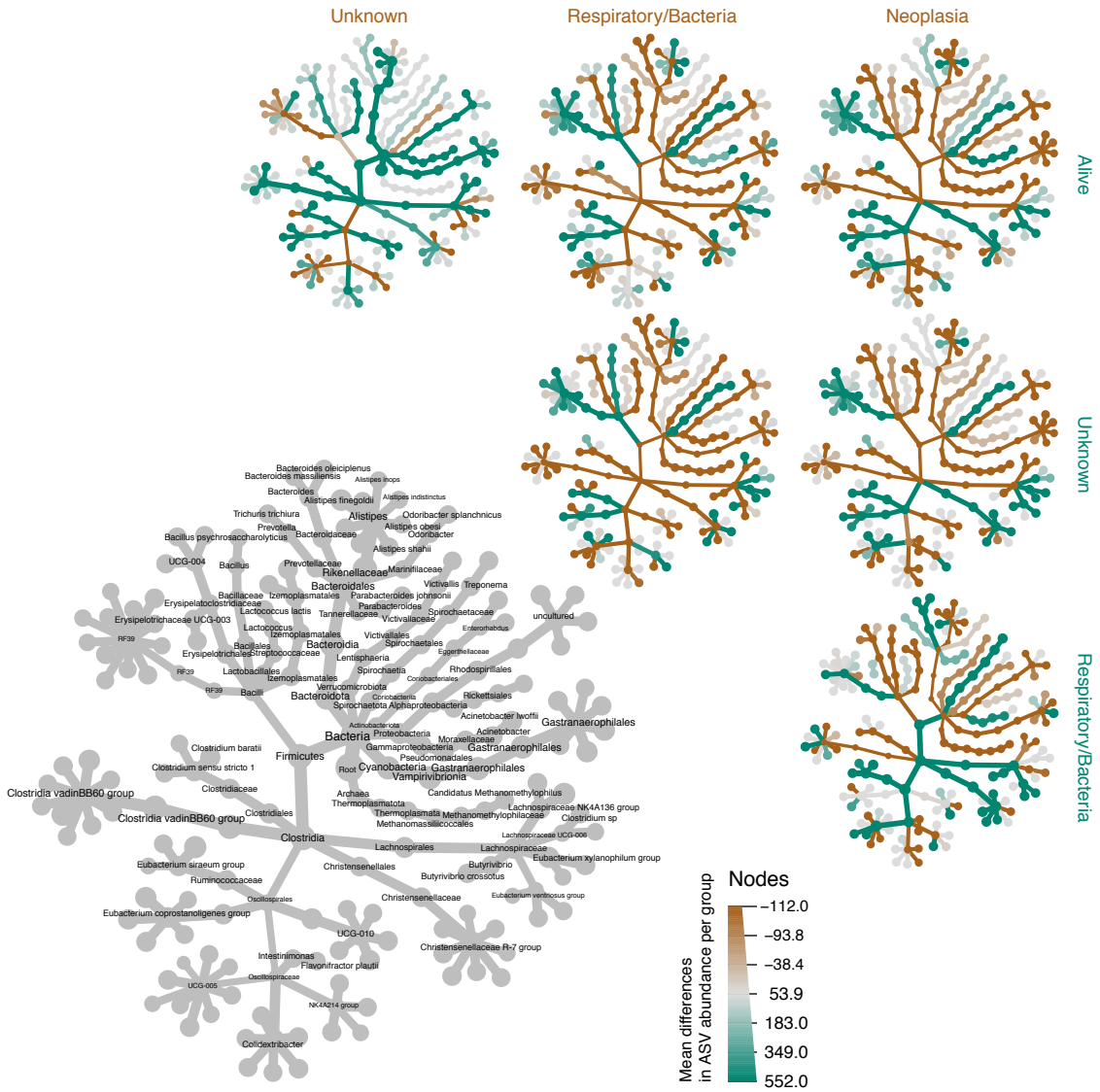

Figure S2. Tree representation of significant LEfSe analysis microbial taxa for VIM source of mortality. Node and edge colors indicate variation in mean pairwise differences in ASV abundance per cause of death. Taxa labels identified as “uncultured” or unassigned” were removed.

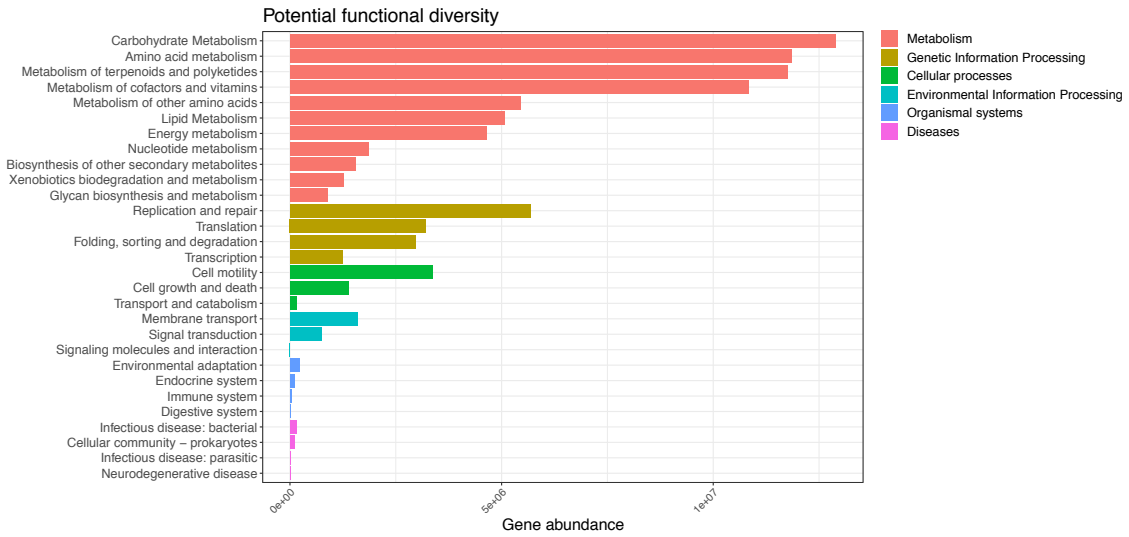

Figure S3. Distribution of gene abundance for KEGG pathways (level 2) categories present in the predicted microbial metagenome of all samples of the VIMs from PICRUSt2. Only categories with an abundance of at least 100 putative genes are shown.

Chapter 3. Gut microbial community variation in pre- and post-hibernation periods in the captive endangered Vancouver Island Marmot (*Marmota vancouverensis*)

3.1 Summary

Captive breeding programs, such as the program for the critically endangered Vancouver Island marmot, include translocations of animals across breeding facilities, both *in* and *ex situ*, and to/from their natural habitat. Records from the Vancouver Island Marmot Recovery Team indicate that newly reintroduced marmots originating from the captive breeding program experience high winter mortality. This health concern may be linked to changes in gut microbiota, which can affect metabolic rate prior to hibernation. Furthermore, captivity itself is known to have a negative effect on the overall abundance of host-derived substrate degraders in the mammalian gut needed for hibernation. In this study, I explored the taxonomical and functional diversity of bacterial communities in the gut of captive and wild-born Vancouver Island marmots before and after the hibernation period, both kept in

captivity at *in situ* and *ex situ* facilities. Housing location impacted the hibernation process by modifying gut microbial communities, as did birth location. Wild-born animals differed in their gut microbial communities compared to other marmots. In the pre-hibernation period, animals kept in the *ex situ* facility seem to have increased abundance in taxa that are metabolically versatile (*Muribaculaceae*, Actinobacteria), compared to more abundant known mucin degraders (Verrucomicrobiota) for *in situ* marmots. For the post-hibernation period, *ex situ* marmots tended to have more taxa associated with fibre degradation than *in situ* conspecifics. Knowing that *in situ* marmots have a gut microbiota similar to wild conspecific, these results confirm the interest to transfer animals held at the zoo at the *in situ* facility before relocation.

3.2 Introduction

The gut microbiota is composed of all the microbial taxa present in the intestine and has several functions such as the degradation and fermentation of carbohydrates and proteins to produce short chain fatty acids (SCFAs), peptides, amino acids and other metabolites, and the synthesis of vitamins (McKenney et al., 2018). It also has immune functions, such as the colonization resistance effect from exogenous pathogens, and antibiotics production (Hooper et al. 2012). Like all organisms, hibernators evolve in close relation with their microbial communities. Hibernation involves extreme physiological changes in mammals (Borbon-Garcia et al., 2017; Bouma et al., 2010; Kurtz et al., 2021), which can affect and are affected by the microbial community (Carey & Assadi-Porter, 2017). Moreover, hibernators like the Vancouver Island marmot (*Marmota vancouverensis*, VIM) fast completely for five to seven months, making them effective models to examine the effects of extreme dietary changes on the resident microbial communities. Previous studies have shown that hibernation induced changes in the microbiota composition and abundance of two ground squirrel species (Carey et al., 2013; Stevenson et al., 2014).

Specifically, hibernation led to a loss of general abundance and diversity in bacteria. Overall, an elevated gut microbial diversity suggests an efficient microbiota because it ensures functional stability in the basic set of biochemical reactions that the microbiota provides to its host (McKenney et al., 2018). During torpor, the abundance of the phylum Firmicutes is particularly reduced, whereas the taxa Bacteroidetes and Verrucomicrobia seem elevated by hibernation in these studies (Carey et al., 2013; Stevenson et al., 2014; Kurtz et al., 2021). Firmicutes are generally considered to be fibre degraders and cross feeders that depend on other microbial taxa and plant material for food degradation (Carey & Assadi-Porter, 2017). However, they usually increase in abundance in the hibernators' gut during the fattening period (post-hibernation), because their growth highly depends on food intake from the host and generally decrease in abundance during fasting (pre-hibernation).

In comparison, the taxa Bacteroidetes and Verrucomicrobia can be considered to be host-derived substrates degraders and most of the SCFAs produced during hibernation result from the metabolism of host-derived secretions (Carey & Assidi-Porter, 2017). Their abundance follows the opposite trend to Firmicutes during pre- and post-hibernation periods. The immune response is also modified during hibernation compared to an active state (Bouma et al., 2010). The adaptive immune system is highly active, as the abundance of lymphocytes is higher in the gut during torpor compared to arousal and active periods. Mucus production in the intestines and colon is sustained as well, and this suggests an elevated microbial activity that appears to be selected by the host for its beneficial functions during fasting.

These studies provided insight on the roles of the gut bacteria during hibernation. However, the impacts of the host's environment are also of interest, and captivity is recognized as a factor in variation of the gut bacterial community in wildlife. Generally, there is a loss in taxonomical diversity and abundance of bacteria in captivity compared to wild counterparts (Borbon-Garcia et al., 2017; McKenzie et al., 2017; Rosshart et al., 2017). As during hibernation, the taxa that change the most in abundance and diversity are the Firmicutes and Bacteroidetes phyla (Cheng et al., 2015; Kong et al., 2014; McKenzie et al., 2017). Firmicutes abundance tends to increase in captivity and Bacteroidetes to decrease compared to wild-reared animals. This could be detrimental to the host if these changes happen in combination with the hibernation-induced microbiota variation. If the Bacteroidetes taxa are not abundant enough, the access to SCFAs degraded from host-derived substrates might not be sufficient. This could be detrimental, as the host depends on SCFAs during torpor. In turn, the abundance of this phylum could decrease gradually from year to year and lead to overwinter mortality. In the end, the redundancy in gut microbial functions that is possible through high taxonomical diversity in the gut microbiota could be impaired from the combination of hibernation and the relocation or birth of the host in captive settings.

Captive breeding programs involve frequent translocations of animals across breeding facilities, between *in* and *ex situ* environments, and the natural habitat of the targeted species. This is the case with the critically endangered Vancouver Island marmot. The free-ranging population was comprised of fewer than 40 individuals in the early 2000s (Lloyd et al., 2019) and the species is now dependent on a captive breeding and reintroduction program, and intensive species management by the Vancouver Island Marmot Recovery Team (VIMRT, Jackson et al., 2015; Roach, 2019; VIMRT, 2017). Individuals are removed from the wild for breeding purposes and are first taken to the *in situ* housing facility on the Vancouver Island, Canada (MRC). They are then moved to *ex situ* facilities such as the Toronto Zoo or the Calgary Zoo to breed. Pups born in one of the zoos are transported to another location to maximize genetic diversity or released back into the wild after a short stay at the *in situ* facility.

The two *ex situ* facilities are situated outside of the natural habitat of the marmots, and are therefore subject to different environmental conditions. The zoos try to minimize any differences in protocols by controlling many aspects of the marmot's living conditions. For example, the temperature is strictly regulated to reflect seasonal fluctuations including natural periods of hibernation. These efforts are focused on one goal: the survival of the marmots through relocation of captive-born marmots in the wild. Records from the VIMRT indicate a higher winter mortality rate of newly reintroduced marmots originating from the captive breeding program than their wild-reared counterparts (Aaltonen et al., 2009; Jackson et al., 2016). These mortality rates could be linked to the animal's food intake after and especially before hibernation. Moreover, the marmots' gut microbial communities may play a role because they are key components of the animal's nutrition and immune response (Trevelline et al., 2019).

Few studies to date examined variation in Gut Microbial Communities (GMCs) between animals present in wild habitat, and *in situ* and *ex situ* facilities within captive breeding programs. I argue that holding animals in captivity within their geographical range might offer greater opportunities for microbial transmission from original substrates, as well as reduced variation in abiotic conditions such as photoperiodism that might mitigate metabolic alteration, influencing circadian rhythms and hibernation (Ren et al., 2020). Physiological variation has already been observed for VIM between *in situ* and *ex situ* facilities. Marmots at the MRC are also known to hibernate longer (on average 24 to 28 days) than at any other *ex situ* locations (Aymen et al., 2021). By comparing wild-born and captive-born animals present in *ex situ* and *in situ* facilities during pre- and post-hibernation periods, we can gain a better understanding of the variation in gut microbiota for the Vancouver Island marmot and identify potential critical links to the poor overwinter survival of the newly released captive-bred marmots.

3.3 Materials and methods

3.3.1 Sample collection and information

All methods were approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University and by the Toronto Zoo Animal Care and Research Committee (ACRC) under the reference 2018-05-02. A total of 214 fecal samples were collected from 16 pairs of VIMs for this study in 2018 and 2019 from two separate locations: the *ex situ* (Toronto Zoo, n=5-6) and *in situ* facilities (Tony Barret Mount Washington Marmot Recovery Center – MRC, n=5-6, Table 1). VIMs in captive housing facilities are paired for mating and pup rearing throughout the year in their enclosures. Captive settings for all VIMs are similar to Aymen et al. (2021). Since animals sharing enclosures usually defecate in the same area, it was

not possible to distinguish which animal the fecal sample originated from, and those samples were therefore treated as belonging to the group of VIMs present in this enclosure. Sample collection was conducted longitudinally according to hibernation dates in the two facilities for two periods: pre-hibernation (fall, September-December) and post-hibernation (spring, April-May). First post-hibernation and last pre-hibernation days were designated as the first/last day animals defecated in the enclosure. Samples were then opportunistically collected from the first day to 29 days (post-hibernation), and 73 days to last day (pre-hibernation) for each pair (Table 1). Fecal samples were collected during daily enclosure cleanings using gloves. They were stored in closed plastic bags in a –20°C freezer until DNA extraction.

A wide range of information was possible to collect and attach to each VIM sampled through the VIMRT and ZIMS: individual sex, age, previous location and date of transfer, and place of birth. Source of mortality was included in the study if the individual died between April and September 2019. Two VIMs died due to respiratory or microbial infection, one from neoplasia and one from unknown causes. Open air enclosure access and presence of pups in the enclosure was also documented according to sampling date. Data for paired VIMs were combined for a number of variables: the minimal age of a group, sex (M/F if pair of the two sexes; M if only males present in enclosure), and locations for place of birth for each individual. For example, if an animal from a pair was born in the wild and another in an *ex situ* facility, the output would be "ex situ and wild".

3.3.2 DNA extraction and sequencing

Gene amplicon sequencing was used to study the bacterial communities. DNA extractions from the fecal samples collected were conducted using the Stool DNA Isolation Kit (Norgen Biotek Corp) following the manufacturer's instructions. Twelve blank extractions were made to control for contamination during the extraction process. A mock community sample (ZymoBIOMICS™ Microbial Community DNA Standard) containing genomic DNA from eight bacterial strains, at 12 ng/µl, was also added in the library to confirm the reliability of our method. After DNA extraction, the targeted gene for taxonomic affiliation (16S rRNA gene) was amplified through PCRs, with one negative control added for sequencing. The library preparation and sequencing were performed by Genome Québec Inc., as well as the demultiplexing of the sequence reads. Using their designated library protocol, 2 × 250 bp with 30,000 reads/sample sequencing was completed using broad bacterial primers of the region V4 of the 16S rRNA gene (515F-806R) using an Illumina NextSeq platform (Illumina Biotechnology Co.).

3.3.3 Bioinformatics

The quality controls of the already demultiplexed paired-end sequence reads were performed through the software FastQC (Andrews, 2010). Sequence reads denoising and amplicon sequence variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al., 2019; v. 2019.1), using the DADA2 pipeline (Callahan et al., 2016) with trimming forward reads to a minimum of 200bp and reverse reads to a minimum of 210bp based on quality scores. ASVs—or also referred to as bacterial phylotypes—were then screened using a pre-trained Naïve Bayes classifier on weighted Silva v.138 99% OTUs full-length sequences (animal distal gut trained dataset, Kaehler et al., 2019) for taxonomical association using the q2-feature-classifier implemented in QIIME2 (Bokulich et al., 2018). Sequence alignment and phylogeny building were also conducted in QIIME2. The mock community sample was removed from the dataset for analysis, after correct identification of 7/8 bacterial strains to the genus level (8/8 family level).

After data importation in R v.4.0.3 (R Core Team, 2018) using the phyloseq package (McMurdie & Holmes, 2013), 40 potential contaminants were identified from the extraction blank from the prevalence-based method using the Decontam package (Davis et al., 2018). Those 40 ASVs were removed from the dataset, as well as well as extraction blank samples, and sequences assigned to mitochondria and chloroplasts for downstream analysis. Trimmed Mean by M-Values (TMM) normalization on raw read counts was conducted on the ASV abundance table instead of sequences rarefaction for beta diversity and differential abundance analyses (McMurdies & Holmes, 2014).

3.3.4 Statistical analysis

Faith's PD, Shannon indices and observed number of ASVs in each sample were used as metrics to measure the α-diversity of gut bacteria between samples. Differences in the index values according to VIM location, place of birth, source of mortality, presence of pups, sex, and days before/after hibernation according to either the pre- or post-hibernation periods, were investigated using restricted maximum likelihood fitting Generalized Additive Models (GAMs). The significance cutoff was set to p-value<0.05 for each test. Homogeneity of variance assumptions and normality of the residuals were inspecting using visual representations through gam.check().

Beta diversity was measured through weighted UniFrac distance matrices between samples (Lozupone et al., 2010). They were used to investigate differences in GMC between the variables VIM environment, sex, parents' birthplace, source of mortality, presence of pups, and days before/after hibernation according to either the pre- or post-hibernation periods using PERMANOVA models Adonis from the *vegan* package were constructed with 9,999 permutations with reported F, R^2, and p-values (Oksanen et al., 2019). Pairwise permutation-

based tests of multivariate homogeneity of group dispersions were then conducted to investigate variations between groups with 9,999 permutations, as well as pairwise PERMANOVAs. Principal component analyses (PCoA) using weighted Unifrac distance measures between samples was conducted to visualize the dissimilarities between groups.

Finally, I used negative binomial distribution for modelling the mapped read counts for each microbial taxa and the semi-parametric SS-ANOVA technique with 1000 permutations for modeling longitudinal profiles of microbial taxa associated with VIM location during the pre- and post-hibernation periods (MetaLonDA, Metwally et al., 2018). After Trimmed Mean by M-Values (TMM) normalization on raw read counts, microbial abundance was aggregated by phylum, family and genus. Only taxa with corrected p-value using the Benjamini-Hochberg method with p-value<0.1 were considered significant.

3.3.5 Metagenome prediction analysis

For metagenome prediction, the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) pipeline was used directly on the ASVs (Langille et al., 2013). This program uses the 16S rRNA gene marker to predict the metagenomic content of a microbial community through sequence inventories available. The output was analysed using the STAMP software keeping unclassified reads for gene frequencies calculations (Parks et al., 2014), through ANOVAs of effect sizes between groups with Games-Howell post-tests and Bonferroni correction for multiple tests, with corrected p-value<0.001 for differences depending on VIM environment.

3.4 Results

34.1 General taxonomy

After reads processing and contaminants filtering, a total read count of 2,971,383 was obtained for gut microbial communities in VIMs, with an average counts per sample of 13,884.97 (SD ± 3,478.127) reads. A total number of 20,893 ASVs – or phylotypes – were identified. During the pre-hibernation period, the Firmicutes phylum was found in greatest relative abundance in both captive VIMs, mainly composed of the *Lachnospiraceae*, *Oscillospiraceae*, *Ruminococcaceae* and *Christensenellaceae* families (*ex situ*: average = 59.53% ± 9.1; *in situ*: 66.02% ± 10.8), followed by Bacteroidota (*Muribaculaceae*, *Bacteroidaceae* and *Rikenellaceae* families, *ex situ*: 29.04% ± 9; *in situ*: 23.17% ± 9.2). The Proteobacteria phylum varied across samples in both group (*ex situ*: 3.5% ± 5.7; *in situ*: 3.93% ± 5.4), as well as the Verrucomicrobiota phylum that is found in greater average proportion in *ex situ* VIMs compared to *in situ* that is mostly represented by *Akkermansiaceae* family (*ex situ*: 3.9% ± 2.2; *in situ*: 2.1% ± 0.9; Figure 1A), and the

opposite pattern for Actinobacteriota. In the post-hibernation period, similar average proportions were found between both captive VIM locations and GMCs were largely dominated by Firmicutes (*ex situ*: 66.3% ± 7.7; *in situ*: 63.2% ± 9.9) and Bacteroidota (*ex situ*: 23.78% ± 6.5; *in situ*: 27.7% ± 8.5), followed by Proteobacteria (*ex situ*: 2.94% ± 1.8; *in situ*: 3.43% ± 4.5) and Verrocumicrobiota (*ex situ*: 2.39% ± 1.8; *in situ*: 2.78% ± 1.6; Figure 1B).

3.4.2 Alpha diversity

When considering the pre-hibernation period, GAMs detected a significant reduction of alpha diversity in animals born in the *in situ* facility approaching hibernation date (MRC, Table 2). While a significant reduction in Shannon index and species richness up to ten days prior hibernation was observable in animals held at the MRC, the Faith's PD index decreased linearly when approaching the start of hibernation for VIM held at the *ex situ* facility (Table 2, Figure 2A). In contrast, the three indices were observed to be significantly lower for VIM at the MRC compared to VIM at the *ex situ* facility throughout the post-hibernation period (Table 2, Figure 2B). More particularly, a linear positive curve was found to best represent the increase in Faith's PD for VIM at the Toronto Zoo and marmots born in the wild had reduced indices as well. Neither the presence of pups or sex had a significant effect on alpha diversity in both periods. However, animals that died of respiratory/infection causes had reduced species richness in the post-hibernation period (Table 2).

3.4.3 Beta diversity

Dissimilarities in GMC composition measured by weighted Unifrac distances for the pre-hibernation were tested using a PERMANOVA model reflecting dissimilarities based on VIM location, explaining 2.7% of variation between GMCs from VIMs *ex* and *in situ* facilities (Adonis: F=2.48, R2=0.027, p-value=0.0088; Table 3) with greater variation in GMCs between *in situ* VIMs than *ex situ* VIMs (betadisper: F=9.7485; p-value=0.0028). While GMCs composition did not vary according to number of days before hibernation (Adonis: F=1.01, R2=0.011, p-value=0.5595), VIMs that died from neoplasia or respiratory/infection causes exhibited different GMC composition (Adonis: F=1.14, R2=0.012, p-value=0.0035; Table 3). However, when considering the post-hibernation period, only VIMs born in the wild exhibited dissimilar GMC composition compared to others (Adonis: F=1.45, R2=0.052, p-value=0.0164), with lower distance to the centroid between samples of wild-born VIMs compared to other birth locations (betadisper: F=4.2601; p-value=0.0032).

3.4.4 Longitudinal differential abundance

Using *MetaLonDA*, dynamics of individual taxonomic features within GMCs at pre- and post-hibernation periods according to VIM location were assessed. At the phylum level, Verrucomicrobiota is noted to establish significant communities for *ex situ* VIMs (p-value<0.05) during early time intervals in the pre-hibernation period (Day 15–19; Figure 4A). Actinobacteria also demonstrate a significant upward trend in the *ex situ* group between days 3 to 13 prior hibernation. During the post-hibernation period, only the Cyanobacteria phylum significantly varied between location (p-value<0.1), being at higher prevalence in *in situ* VIMs from day 1 to 3 and decreasing onwards, while it increased and became higher in prevalence in *ex situ* VIMs after day 4. At the family level, all significant taxa were in higher proportion in *ex situ* VIMs for the pre-hibernation period, belonging to the Firmicutes and Spirochaetota phyla, apart from the *Muribaculaceae* (Bacteroidota) that was enriched for *in situ* VIMs from day 6 to 16 (Figure 4B). For the post-hibernation period, all three Firmicutes families significantly increased in abundance over time in *ex situ* VIMs, in opposition with *Flavobacteriaceae* (Bacteroidota) that increased in *in situ* VIMs (Figure 4C).

At the genus level, five genera from the Firmicutes phylum and one from Bacteroidota (*Bacteroides* sp) were enriched in the pre-hibernation period in GMCs of *ex situ* VIMs, for five genera of Firmicutes, two from Bacteroidota (*Bacteroides* and *Rikenellaceae RC9 gut group*) and two of Proteobacteria (*Escherichia-Shigella* and *Parasutterella* sp) in GMCs of *in situ* VIMs (Figure 4D). From those, the *Ruminococcus* and *Bacteroides* genera were enriched for *in situ* VIMs when approaching the date of hibernation, while it was enriched in *ex situ* VIMs at earlier dates (Figure 4D). The same pattern is observable during the post-hibernation period for three genera: *Paraeggerthella* (Actinobacteriota), *Eubacterium* (Firmicutes), and *Angelakisella* (Firmicutes); while an opposite pattern was found for the *Lachnospiraceae NK4A136 group* (Firmicutes; Figure 4E). For GMCs of *ex situ* VIMs, an increase of four Firmicutes genera, one Verrucomicrobiota (*Victivallis* sp), one Proteobacteria (*Methylobacterium-Methylorubrum* sp) and one Actinobacteriota (*Paraeggerthella* sp). On the other end, GMCs of *ex situ* VIMs were enriched in four Firmicutes genera and *Paraeggerthella* as well, but also two Bacteroidota genera (*Butyricimonas* and *Porphyromonas* sp; Figure 4E).

3.4.5 Metagenome prediction

I examined differences in microbial gene abundances by KEGG pathways according to VIM environment. During the pre-hibernation period, genes related to metabolism differed between groups, with increased abundance of genes for amino acid metabolism and xenobiotics degradation and metabolism in GMCs of VIMs located at the MRC, while VIMs in the *ex situ* facility had more microbial genes related to glycan, secondary metabolites as well as terpenoids and polyketides metabolism (Figure 5). In the post-hibernation period, VIMs at the MRC were enriched in gut microbial genes involved in riboflavin metabolism (Eta2=0.246;

p-value<0.001) and marmots at the Toronto Zoo with butanoate metabolism (Eta2=0.151; p-value<0.001).

3.5 Discussion

Overall, the gut microbiota of VIMs in the pre- and post-hibernation periods exhibited differences according to captive settings, even though the dominance of the Firmicutes and Bacteroidota phyla was similar to other hibernating rodents (Carey & Assadi-Porter, 2017). According to alpha diversity analysis, bacterial species richness decreased when approaching entry into hibernation, which is consistent with an increase of alpha-diversity during hibernation in other mammals (Dill-McFarland et al., 2014; Stevenson et al., 2014). A reduction of species diversity has been documented during the hibernation period (reviewed in Carey & Assadi-Porter, 2017), as GMCs adapt to the altered physical and metabolic environment brought on by hibernation. Similarly, emergence from hibernation is a transitional period during which the GMC adapts to metabolic changes with increases in species diversity. However, I observed differential trends in the pre-hibernation period between VIM locations. While *ex situ* VIMs experienced an overall linear loss of species diversity, a polynomial trend was observed for *in situ* VIMs. As documented by Aymen et al. (2021), wild-born and *in situ* VIMs are known to hibernate longer (24-28 days) than *ex situ* VIMs, where the closest approximation between captive and natural hibernations was noted at MRC (Bryant & McAdie, 2003). This is consistent with the results of the previous chapter, where VIMs located at the MRC had similar GMCs to free-ranging VIMs compared to captive conspecifics held at *ex situ* facilities. In that sense, *in situ* VIMs might experience a loss in microbial diversity similar to the trends during the natural pre-hibernation period, while different dynamics seem to occur for *ex situ* VIMs. On the other hand, positive linear trends were observable for both VIM locations during the post-hibernation period. However, we see an overall greatest species richness for *ex situ* VIMs in this period. While an increase in microbial diversity has been observed in hibernators upon emergence (Carey & Assadi-Porter, 2017), species richness is not a common trend in captive mammals compared to semi-wild and wild conspecifics (Borbon-Garcia et al., 2017; Tang et al., 2020; McKenzie et al., 2017). These results provide first evidence that captive settings can impact GMCs during the hibernation process. However, the ultimate causes of microbial diversity increase remain unknown, and could be linked to higher and differential food intake for *ex situ* VIMs, but shortage in water availability and the nutritional composition of food could also be proximate drivers of hibernation regulation (Siutz et al., 2017; Vuarin & Henry, 2014). Moreover, because *ex situ* VIMs experience shorter hibernation periods (Aymen et al., 2021), it is possible that microbial dynamics during artificial hibernation are not as reduced as natural hibernation due to physical and metabolic variation according to environmental factors between captivity and the natural habitat (Kohl et al., 2014; Sonoyama et al., 2009).

For beta diversity analysis, I observed dissimilarities in GMCs according to VIM location and birthplace rather than time variation. Those results are similar to those described in the previous chapter, that could be potentially linked with variation in diet between facilities for the VIMs during the active season, as well as early microbial colonization linked to birth location (Metcalf et al., 2017; Spor et al., 2011). However, some taxa were longitudinally differentially abundant between VIM locations in both pre- and post-hibernation periods. I hypothesized that abundance of the Firmicutes and Bacteroidota phyla would differ in both periods according to VIM location. Although most of the differential taxa belong to both phyla, it is important to acknowledge taxa variation at different taxonomical levels.

At the phylum level, I observed a significant reduction in the abundance of Verrucomicrobiota in the early pre-hibernation period for *ex situ* VIMs, while this phylum tends to be more abundant in hibernating phases than during the active season (Carey & Assadi-Porter, 2017; Kurtz et al., 2021), due to the specialization in the degradation of host-derived substrates, such as mucin glycoproteins (Derrien et al. 2004). Given that the PICRUSt analysis showed a greater abundance of genes involved in glycan biosynthesis and metabolism in *ex situ* VIMs, it is possible that other alternative taxa mitigate the loss of beneficial functions from Verrucomicrobiota in host-derived substrates degradation. For example, the *Bacteroides* genus is known to use either dietary or host-derived glycans depending on availability (Raimondi et al., 2021), and the versatility of the *Muribaculaceae* family and thus the ability to occupy different niches in microbial communities (Lagkouvardos et al., 2019; Ormerod et al., 2016). However, there is still uncertainty about the genetic potential of GMCs given the approximate nature of metagenome predictions. Other taxa enriched in *ex situ* VIMs that are poorly documented in terms of functions within GMCs could also take up this role, such as the *Acholeplasmataceae* and *Selenomonadaceae* families.

On the other hand, Actinobacteria were enriched in *ex situ* VIMs during the pre-hibernation period, a phylum that is known to decrease in abundance during hibernation for other mammals (Sommer et al., 2016; Dill-McFarland et al., 2014). This phyla is known to produce a high concentration of acetate that can protect the host from entero-haemorrhagic and mucin degraders *Escherichia coli* and *Shigella* (Fukuda et al., 2012; Raimondi et al., 2021), genera enriched in *in situ* VIMs compared to *ex situ*. Moreover it has been demonstrated that Actinobacteria have a non-negligible production of lactate, which can be metabolized by a group of bacteria to produce butyrate (Scott et al., 2014). In this context, butyrate abundance is correlated with an increased expression of the gene MUC2 (Gaudier et al., 2004), a mucin glycoprotein that is one of the major components of the mucous layer that can be degraded by other bacteria such as *Bacteroides* and *Muribaculaceae*.

Within the same pre-hibernation period, several taxa enriched in *ex situ* VIMs have been associated with captivity in other mammals. For example, the *Ruminiclostridium* genus is also in greater abundance in the GMCs of captive Père David's deer compared to free-ranging conspecific, although their function in the gut in poorly understood (Sun et al., 2019). In the same way, the *Spirochaetaceae* family was documented in greater abundance in captive primates (Clayton et al., 2017), pigs (Correa-fiz et al., 2019) and other mammals (McKenzie et al., 2017). In naked mole rats particularly, the *Treponema* genus from this family is found in captive individuals for its ability to digest and extract valuable nutrition from fibrous plants (Debebe et al., 2017). Another taxa linked with fibre degradation is the *Ruminococcus* genus (David et al., 2014), also found in greater proportion in *ex situ* VIMs. This genus is known to increase during the active season for other hibernators (Stevenson et al., 2014) and to decrease during fasting for tetrapods in general (Kohl et al., 2014). Given that many taxa were differentially abundant between VIM locations and seem to be linked to fibre and host-derived substrates degradation, it is possible that the pre-hibernation period GMCs dynamics differ according to dietary input. To further investigate the impacts of diet on the GMCs of the marmots compared to geographical locations, a diet manipulation within facilities with raw foods would allow to understand its impact on GMCs variation. Because the pre-hibernation period is especially critical for reintroduced captive-bred marmots (Jackson et al., 2016), it is important to understand further if the gut microbiota can be modulated through diet change to mimic wild conditions for hibernation success.

When considering the post-hibernation period, I observed an opposite pattern between *in situ* and *ex situ* VIMs. *Ex situ* VIMs are enriched in *Monoglobaceae* and *Eubacterium* families, that are known to be mucin degraders in the human gut (Raimondi et al., 2021), therefore thriving through host-derived substrates. On the other hand, *in situ* VIMs have greater abundance of *Butirycimonas* that are not considered mucin degraders (Raimondi et al., 2021), as well as *Porphyromonas* that are known to increase in later winter for hibernators (Dill-McFarland et al., 2014) and to digest plant polysaccharides in rodents (Carey & Assadi-Porter, 2017). It is possible that glycan degradation is more intense in the gut *ex situ* VIMs, while shifts for the active season are taking place more rapidly for GMCs of *in situ* VIMs. Longitudinal variation in other taxa such as the *Christensenellaceae* family are more difficult to interpret, as they are associated with gut health (Waters & Ley, 2019), and early increase in abundance upon emergence for *in situ* VIMs compared to *ex situ* VIMs could be beneficial after microbial loss during hibernation. It is worth noting that many taxa enriched in both marmot groups remain poorly understood, such as the *Victivallis*, *Anaerofustis* and *Facklamia* genera.

Overall, this study provides a first look at the GMCs of the Vancouver Island marmot around the hibernation periods. I have shown that host location can be driver of GMCs in captive marmots, and because of the low overwinter survival in captive-released marmots (Jackson et

al., 2016), it is possible that gut microbial metabolic pathways fostered in captivity might not be adequate for hibernation in the natural habitat. For this reason, further omic approaches would allow us to understand the metabolic importance of the marmots' gut microbiota, especially during hibernation between the two types of captive settings. Microbiome biology is still a poorly explored field in conservation biology and has an enormous potential for elucidating the effects of captivity maintenance conditions on host health. Microbiome analyses could be a powerful tool for government policy makers, improving the current management plans of emblematic and threatened wild species such as the Vancouver Island Marmot, whose populations have been reduced and little is known about the current diet and potential implications for hibernation success.

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3.8 Tables and figures

Collection period	Location	Number of VIM pairs	Number of samples (Min-Max by VIM)	Temporal range (days) before and after hibernation
Pre-hibernation (fall)	*In situ* - MRC	5	50 (10)	1-73
	Ex situ - Toronto Zoo	6	55 (6-10)	1-32
	Sub-total	11	105	1-73
Post-hibernation (spring)	*In situ* - MRC	6	59 (9-10)	1-17
	Ex situ - Toronto Zoo	5	50 (10)	1-29
	Sub-total	11	109	1-29

Table 1. Information on fecal sample collection according to location of VIM pairs, with number of minimal and maximal number of samples collected by VIM.

(A) Pre-hibernation

Shannon	Predictor	Estimate	Std. Error	t-value	Pr(>|t|)
De=31.1%	Place of birth: in situ	-0.22262	0.10650	-2.090	0.040
	Smooth terms	Edf	Ref.df	F	p-value
	Days before hibernation:location in situ	2.678	3.314	3.504	0.0188
Observed	Predictor	Estimate	Std. Error	t-value	Pr(>|t|)
De=36%	Place of birth: in situ	-30.251	10.890	-2.778	0.00695
	Smooth terms	Edf	Ref.df	F	p-value
	Days before hibernation:location in situ	2.732	3.383	3.637	0.0162
Faith's PD	Predictor	Estimate	Std. Error	t-value	Pr(>|t|)
De=41.4%	Place of birth: in situ	-4.4354	1.2691	-3.728	0.000376
	Place of birth: wild	-3.9739	1.2872	-3.087	0.002842
	Smooth terms	Edf	Ref.df	F	p-value
	Days before hibernation:location ex situ	1.000	1.000	4.999	0.0284

(B) Post-hibernation

Shannon	Predictor	Estimate	Std. Error	t-value	Pr(>|t|)
De=45.4%	Location: in situ	-0.46619	0.14916	-3.125	0.00244
Observed	Predictor	Estimate	Std. Error	t-value	Pr(>|t|)
De=46.8%	Location: in situ	-55.473	15.903	-3.488	0.000777
	Mortality: Respiratory/Infection	-19.875	8.483	-2.343	0.021492
Faith's PD	Predictor	Estimate	Std. Error	t-value	Pr(>|t|)
De=56.4%	Location: in situ	-4.2998	1.6719	-2.572	0.0119
	Place of birth: wild	-3.9439	0.6971	-5.657	2.09e-07
	Smooth terms	Edf	Ref.df	F	p-value
	Days after hibernation:location ex situ	1.000	1.000	3.074	0.0452

Table 2. General Additive Models results of restricted maximum likelihood fitting for the two datasets (A: pre- and B: post-hibernation periods) investigated according to predictor variables of interest for three alpha diversity indices. Presented values are meeting the significance cutoff of p-value<0.05. De: Deviance explained by each model.

Dataset	Variable	Level1	Level2	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Pre-hibernation	VIM environment	in situ	ex situ	6.3330e-08	6.3330e-08	2.48040	0.0278	0.0088
	Mortality	alive	respiratory/infection	4.5010e-08	4.5015e-08	1.8127	0.0193	0.01099
		alive	neoplasia	4.1000e-08	4.1001e-08	1.6185	0.0191	0.02897
Post-hibernation	Birthplace	in situ & wild	wild	1.3137e-08	1.3137e-08	2.2609	0.08	0.00299
		ex situ & wild	wild	1.1680e-08	1.168e-08	1.889	0.0677	0.00199
		ex situ	wild	9.4900e-09	9.4859e-09	1.5131	0.0263	0.02098
		in & ex situ	wild	1.2230e-08	1.2231e-08	1.6999	0.0364	0.01499

Table 3. Pairwise PERMANOVA models for significant variables (p-value<0.05) in overall PERMANOVA models for weighted Unifrac distances between samples in pre- and post-hibernation periods. Only values meeting the significance cutoff of p-value<0.05 are represented.

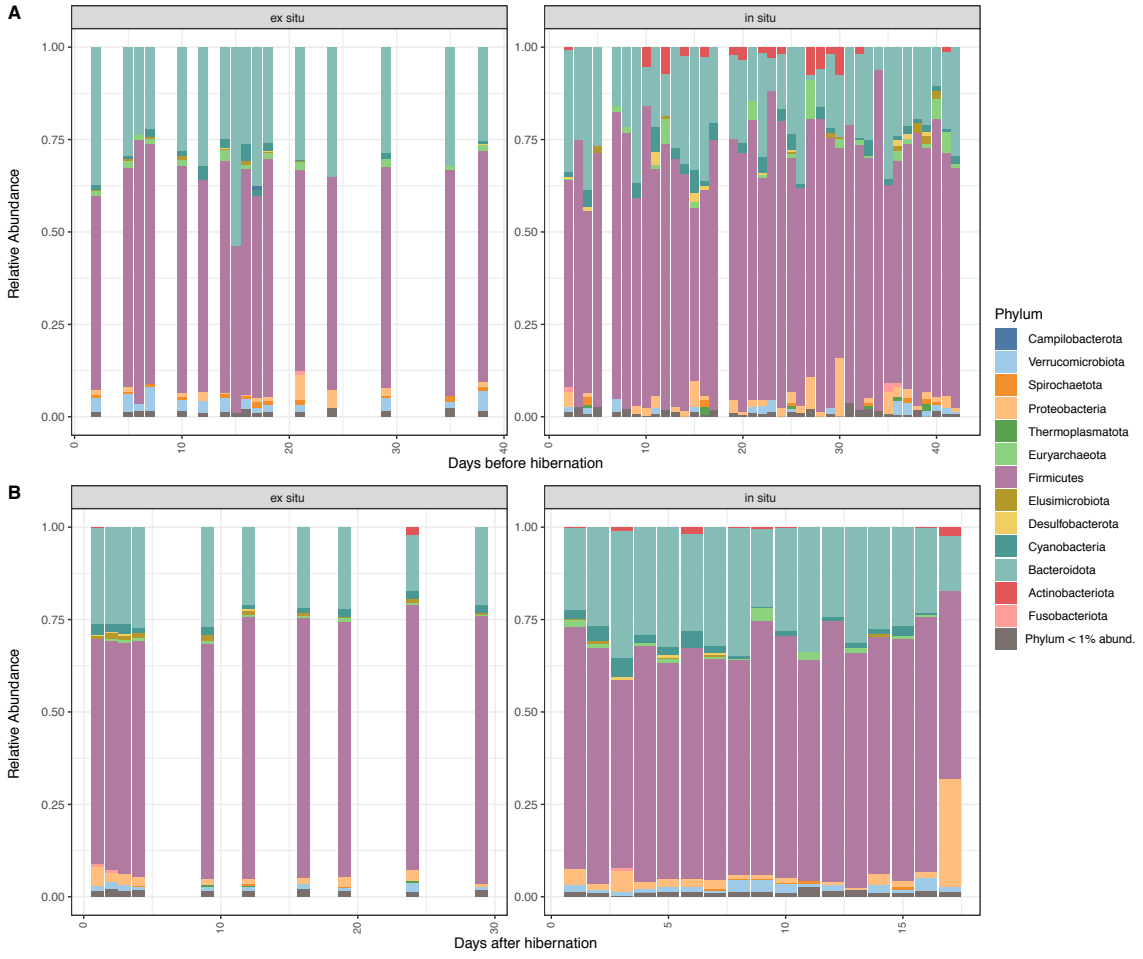

Figure 1. Mean relative abundance of major microbial phyla recovered from *M. vancouverensis* fecal samples located in the different captive facilities (*in situ* location and *ex situ* location) by date (A) before hibernation, and (B) after hibernation.

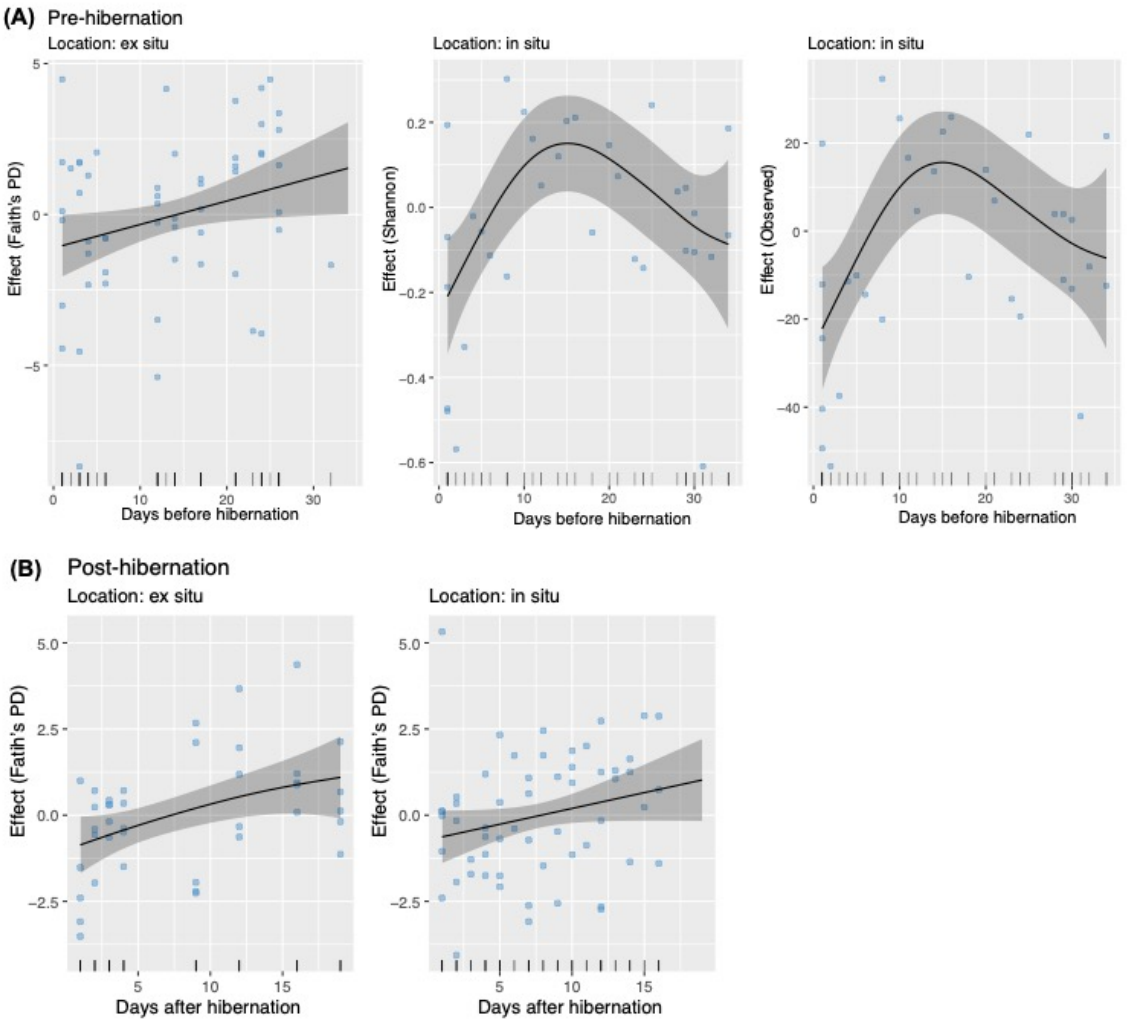

Figure 2. Generalized additive model (GAM) plots showing the partial effects of VIM location on alpha diversity indices during (A) pre-hibernation and (b) post-hibernation periods. The tick marks on the x-axis are observed data points. The y-axis represents the partial effect of each variable with index investigated specified. The shaded areas indicate the 95% confidence intervals.

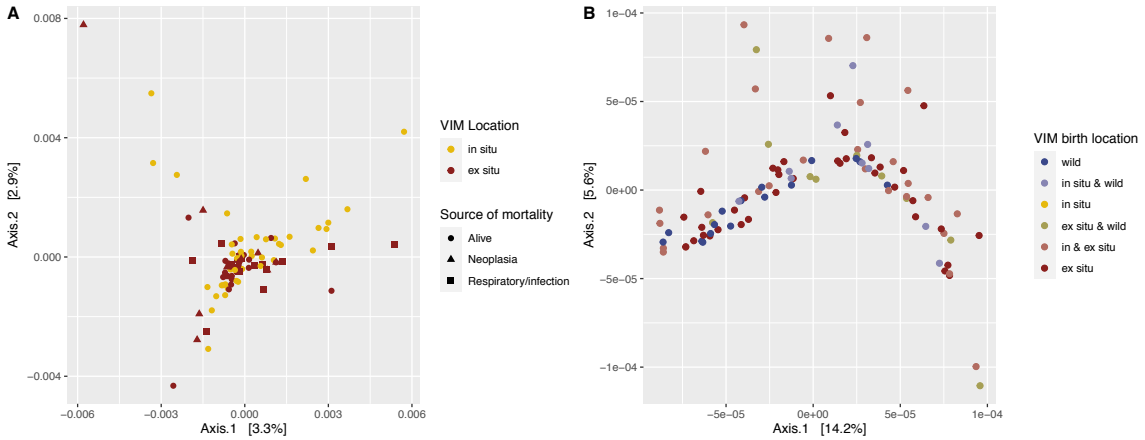

Figure 3. PCoA on weighted Unifrac distances between samples for (A) pre-hibernation period according to VIM location and source of mortality and (B) post-hibernation period according to VIM birth location.

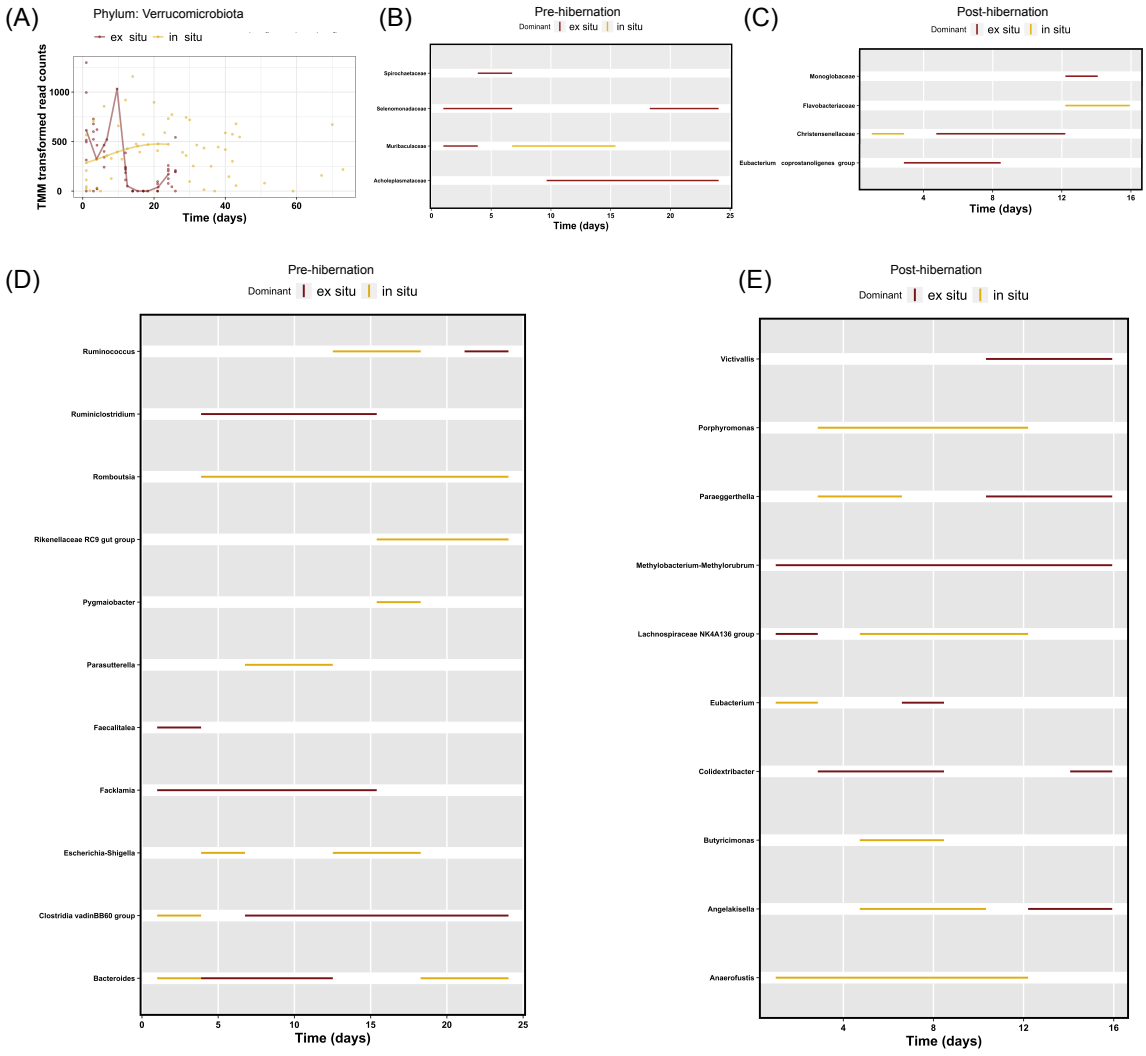

Figure 4. Longitudinal differential abundance (TMM transformed read counts) of microbial taxa (A: phylum, B&C: family; D&E: genus level) according to VIM location (ex situ and in situ) during the pre- and post-hibernation periods. 0 on the x-axis corresponds to the day of entrance (pre-hibernation) or emergence (post-hibernation) from hibernation. Only taxa meeting the significance cutoff of p-value<0.1 are represented.

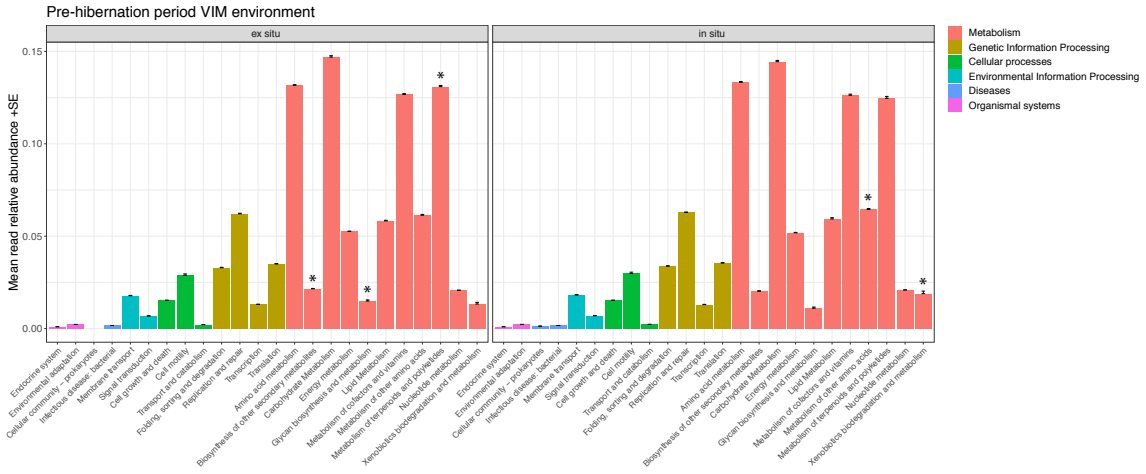

Figure 5. Mean relative abundance (with standard errors) of predicted microbial genes according to KEGG pathways (level 2) by VIM environment. * represents significant post-hoc test for increased abundance for this pathway in the specific VIM group. Only categories with a mean abundance of at least 0.001 are shown.

Chapter 4. A microbial tale of farming, invasion and conservation: on the gut bacteria of European and American mink in Western Europe

4.1 Summary

One of the threats that the critically endangered European mink (*Mustela lutreola*) faces throughout its relict range, including the occidental population, is the impact of the American mink (*Mustela vison*) invasion in its natural habitat. I aimed to explore the differences in microbiota and genetic diversity between European and American mink to test phylosymbiosis theory. I investigated the gut microbiota composition of European and American mink in a controlled environment (captive breeding compounds and fur farms

respectively) to account for the impact of the environment on gut bacterial composition. I compared them to the gut microbiota of both mink species in the natural environment across multiple habitats. Our exploratory results showed differences between free-ranging and captive individuals, with more extreme changes in American mink compared to European mink. However, feral American mink from a long-established population exhibited gut bacterial composition closer to the free-ranging native species compared to more recently established feral populations. This result could be explained by dietary shifts in the area sampled based on prey availability through different landscape, but also to a lesser extent due to greater genetic differentiation. This exploratory work contributes to the scarce literature currently available on the dynamics between gut microbiota and mammal invasion.

RESEARCH ARTICLE

A Microbial Tale of Farming, Invasion and Conservation: On the Gut Bacteria of European and American Mink in Western Europe

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4.2 Introduction

Invasive alien species have been widely recognized as one of the major threats to biodiversity due to anthropogenic changes at both global and local scales (Lockwood et al., 2007). Invasive species can directly impact the habitat and ecology of native species they interact with as they affect native species' population sizes and habitat ranges (Genovesi et al., 2012; Zalewski et al., 2010). An example of such a successful invader is the American mink (*Mustela vison*) in Europe, which was introduced from North America for fur farming in the early 20th century. Following accidental escapes, as well as intentional releases, the mink became established in 28 European countries (Bonesi & Palazon, 2007; Reid et al., 2016). This species is also present as an invasive species in parts of South America and Asia (Mora et al., 2018; Shimatani et al., 2010).

The generalist and opportunistic aspects of this mustelid's diet strongly impacted populations of 47 reported native species, reducing prey species of seabirds (Nordström et al., 2002), voles

(Banks et al., 2005), and crustaceans (Fischer et al., 2009), six of them being included in the IUCN Red List categories near threatened, vulnerable, endangered and critically endangered (Genovesi et al., 2012). One of them is the critically endangered European mink (*Mustela lutreola*), with evidence of direct aggression from the invader towards the native species involved in competition for resources (Melero et al., 2008; Sidorovich et al., 2010; Podra et al., 2013). Both species have similar ecological niches, being carnivorous mammals in riparian ecosystems and predating on both aquatic and terrestrial prey. The presence of the American mink in the native species habitat was shown to reduce the diet of European mink so that it becomes more specialized, while the American mink's diet became more generalist (Sidorovich et al., 2010).

The American mink can also play a role in disease transmission among native species, as they can carry the Aleutian Disease Virus (ADV), the Canine Distemper Virus (CDV) as well as many eukaryote parasites that can be transmitted to other mustelids, feral cats, and even humans (Leimann et al., 2015; Martínez-Rondán et al., 2017; Torres et al., 2007). Studying the invasive success of a carrier species like the American mink becomes even more critical, especially because mink, feral and/or in farms, interact with many other species and humans.

In France, the American mink was introduced in the 1920s in the Eastern side of the country; in the 1950s, many farms moved to the western side where access to fish by-products for mink feeding was readily available (Léger et al., 2005). A long-term monitoring study from 2000 to 2015 recorded evidence of the expansion of the American mink over the Atlantic coast in France with multiple established populations, including: (1) the historical region of Brittany, Normandy and Pays de la Loire, (2) the western region of the Pyrenees up to northern Aquitaine, and (3) the Eastern region of the Pyrenees (Léger et al, 2018). In contrast, the western distribution of the European mink is reduced to seven departments of southwestern France (Maizeret et al., 2002) and to northern Spain, mainly in Navarre, La Rioja and some neighbouring communities in the Upper Ebro Basin (Põdra & Gomez, 2018). Moreover, French populations are probably highly fragmented, especially in departments where the invasive species is abundant.

The low density of individuals in these regions and low genetic diversity of the Western population perhaps due to a bottleneck event (Cabria et al., 2015; Michaux et al., 2005) encouraged the creation of a captive breeding program in Spain at the Fundación para la Investigación en Etología y Biodiversidad (FIEB), with individuals originating from free-ranging populations in Spain.

Before a species establishes and expands to become a successful invader, the colonization of new habitats represents a challenge through a variety of new selective pressures encountered

that can be highly costly from an adaptative lens. Therefore, host-associated microbes can play a critical role in the invasive success of an exotic species in a new habitat. These microorganisms (bacteria, archea, virus, fungi and protozoa) range from parasites to obligate mutualists (McKenney et al., 2018; West et al., 2019). This large range of interactions, often coupled with complex historical and introduction events, can result in a wide variety of ecological dynamics.

Within the last decade, we have begun to understand the underlying processes driving host-associated microbial community dynamics. The external environment of the host has been reported to be one of the main drivers of variation (Koskella et al., 2017; Spor et al., 2011). Housing facilities such as fur farms and captive breeding facilities in zoos provide intense veterinary care, sanitized enclosures, a standardized diet, and reduced social interactions. Hence, captivity has been shown to alter the microbiota of animals compared to their free-ranging counterparts (Clayton et al., 2016; Wasimuddin et al., 2017; van Leeuwen et al., 2020). The majority of these studies show similar trends: a decrease in bacterial phylotype richness (or α-diversity) among captive individuals compared to their free-ranging conspecifics, as well as differences in community composition (or β-diversity) between the groups. However, some host species show the opposite pattern (McKenzie et al., 2017; Greene et al., 2019; Frankel et al., 2019), postulating that the gut microbiota respond differently to captivity according to host taxa, mainly through their feeding strategy and gut physiology. Differences observed in gut microbial communities have largely been attributed to altered diets in captivity that can also lead to the extinction of microbial niches and functions in the host's gut over multiple generations in captivity (Sonnenburg et al., 2016; van Leeuwen et al., 2020). Diet has therefore been reported to be the most important influence on the mammalian gut microbiota (Reese & Dunn, 2018; Martinez-Mota et al., 2019).

Despite the strong impact of the host environment on its gut microbial community, the genetics and biology of the host should also be taken into account to fully understand the complex dynamics that occur in these systems (Koskella et al., 2017; Spor et al., 2011). Phylosymbiosis is described as an increase in compositional similarity between bacterial communities colonizing closely related hosts compared with distantly related hosts (Groussin et al., 2017; Lim & Bordenstein, 2020). Many investigated mammals have supported this pattern, such as bats, apes and rodents (Brooks et al., 2016; Ochman et al., 2010; Kohl et al., 2018; Knowles et al., 2019), as well as other animal taxa (Pollock et al., 2018; Sevellec et al., 2019; van Opstal & Bordenstein, 2019); however, other studies have not detected signals of phylosymbiosis in some mammals (Baxter et al., 2015; Greene et al., 2019; Grond et al., 2020). Groussin et al. (2017) also suggested that the tight associations between some host taxa and some of their associated gut microbes might not generalize to the entire gut microbial community, hence the strong environmental effects on gut microbial composition. No study

to date has examined phylosymbiosis in the context of invasion ecology in carnivores. Carnivores have short transit time and digestive tracts, so the gut microbiota are potentially less impacted by diet (Reese & Dunn, 2018; Ley et al., 2008). From the current literature, mustelids are known to harbor relatively low diversity and abundance of gut microbes (Compo et al., 2018; Bahl et al., 2017). Moreover, large interindividual variation in gut bacterial communities' composition has been observed in farmed American mink, being generally dominated by the phylum *Firmicutes*, but in some cases also *Proteobacteria* and *Fusobacteria* (Compo et al., 2018; Bahl et al., 2017). At the class level, the average bacterial composition was dominated by *Clostridia*, *Gammaproteobacteria*, and *Fusobacteria*.

The purpose of this study was to understand the relationships between the gut microbiota of related invasive and native host species (specifically European and American mink) sharing similar ecological niches. I was interested in: (i) if the environment (free-ranging or captive) had a stronger influence than species or population identity for gut microbial diversity and composition, (ii) if there were fewer differences in abundance of microbial taxa within mink species than between them across multiple populations, and (iii) if genetic relationships between host populations were reflected in terms of gut microbial compositional similarity. To study these questions, I examined gut bacterial species (or phylotype) richness, gut microbiota structure, and composition differences between American and European mink in captive settings (fur farm and captive breeding program), and in the natural environment across three different habitats in western France and Spain (Brittany region, the Nive basin in the Pyrénées-Atlantiques departement, and Navarra). To test for a phylosymbiosis signal, I also investigated the genetic diversity and structure of those mink populations using previously collected data through neutral microsatellite markers analysis.

4.3 Methods

4.3.1 Sample collection and study sites

Fecal samples and rectal contents were collected from live or dead animals from five different populations. For free-ranging populations, six European mink were sampled in the Navarra region (Spain), twelve American mink were sampled in the Nive watershed (Southwest, Pyrénées-Atlantiques, France) and sixteen American mink from Brittany (Tomé island and close mainland; Figure 1). In order to investigate habitat variation from each free-ranging sampled populations, a map was created using QGIS 3.16.6-Hanover with GPS coordinates for each sample. Layers documenting landscape use, were simplified to agricultural, built, natural and water surfaces from datasets originating from IDENA (Spatial Data Infrastructure of Navarre) and data.gouv.fr from Open Street Map (Alexandre Lexman). For captive populations, ten European mink were sampled in captive settings at the Fundación

para la Investigación en Etología y Biodiversidad breeding center (FIEB) and fourteen American mink from a fur farm in the Pyrénées-Atlantiques department (Southwest of France). All samples were collected using sterile tweezers and placed in sterilized microcentrifuge tubes filled with 96% ethanol. Those tubes were stored in a -20°C freezer until further processing (Asangba et al., 2019).

4.3.2 DNA extraction and sequencing

Gene amplicon sequencing was used to study the bacterial communities. DNA extractions from the fecal samples collected were conducted using the QiaAmp Mini Kit with Inhibitex (Qiagen) following the manufacturer's instructions. Two blank extractions were made to control for contamination during the extraction process. A mock community sample (HM-783D, BEI resources) containing genomic DNA from 20 bacterial strains at concentrations ranging from 0.6 to 1400 pg/µl was also added in each library to confirm the reliability of our method. After DNA extraction, the targeted gene for taxonomic affiliation (16S rRNA gene – 515F & 806R) was amplified through polymerase chain reactions (PCRs). The library preparation and sequencing were performed by Novogene UK. Using their designated library protocol, 2 × 250 bp paired-end sequencing was completed using broad bacterial primers of the region V4 of the 16S rRNA gene using an Illumina NovaSeq platform in 100k reads/samples depth (Illumina Biotechnology Co.).

4.3.3 Bioinformatics

The quality controls of the paired-end sequence reads were performed through the software FastQC (Andrews, 2010). Sequence reads demultiplexing, denoising and amplicon sequence variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al., 2019; v. 2020.8), using the DADA2 pipeline (Callahan, McMurdie, & Holmes, 2017; Callahan et al., 2016). ASVs –or also referred to as bacterial phylotypes– were then screened to the 97% 16S rRNA gene full-length reference sequences from the Silva RDP v.138.1 database (Pruesse et al., 2007) for taxonomical association using the VSEARCH classifier implemented in QIIME2 (Bokulich et al., 2018). Sequence alignment and phylogeny building were also conducted in QIIME2.

Analysis of a negative control showed the presence of bacterial sequences that probably derived from contamination during laboratory sample handling. However, the diversity of this control was dissimilar from those of all mink samples (Bray-Curtis dissimilarity > 70.8%). For subsequent analysis of sequences associated with mink samples, negative control sequences were trimmed from the whole dataset. The cumulative sum scaling (CSS) method was used to normalize the data using the *metagenomeSeq* package (Paulson et al., 2013) in R (R

version 3.5.2, R Core Team, 2018). It can decrease the fold difference in sampling depth and avoid the rarefying of counts (Weiss et al., 2017).

5.3.4 Statistical analysis for comparison of α-diversity of gut bacteria between groups

After CSS normalization, mink groups were divided as followed: European mink in captivity (EM Breeding Center; n=7), American mink in captivity (AM Farm; n=14), free-ranging European mink (EM Spain; n=6); and free-ranging American mink distinct populations in Brittany and Nive (AM Brittany; n=15 and AM Nive; n=10; Figure 1). All statistical analyses were conducted in R (R version 3.5.2, R Core Team, 2018) using the *phyloseq* (McMurdie & Holmes, 2013) and *microbiome* packages (Lahti, 2017) for manipulation of data. Chao1, Shannon indexes and Faith's PD in each sample were used as metrics to measure and compare the α-diversity of gut bacteria between groups. Chao1 is an indicator for overall bacterial species richness, the Shannon index characterizes both the abundance and richness of bacterial phylotypes, and Faith's PD is a measure for phylogenetic diversity. Differences in the index values according to mink population, host species, host environment (wild or captive settings) and sex were investigated using a non-parametric Kruskall-Wallis rank sum test followed by Dunn test (1964) of Kruskal-Wallis multiple comparisons with Benjamini & Hochberg (1995) for p-value correction. The significance cutoff was set to p-value<0.05 for each test.

4.4.5 Statistical analysis for comparison of β-diversity of gut bacteria between groups and differential abundance

Unweighted and weighted UniFrac distance matrices between samples (Lozupone et al., 2010) were used to investigate differences in gut microbial communities between population, host sex, host environment, and host species with all bacterial taxa. A PERMANOVA model Adonis from the *vegan* package was constructed with 9,999 permutations with reported F, R2, and p-values to determine whether there were significant differences between the mink populations, host species, and sex as main effects (Oksanen et al., 2019) after testing the homogeneity of groups variances using the *betadisper* function from the same package. Pairwise PERMANOVAs were then conducted to investigate variations between groups with 9,999 permutations. A principal component analysis (PCoA) using Unifrac distance measures between samples was conducted to visualize the potential similarities between groups. Finally, a UPGMA dendrogram was constructed using the *qiime diversity beta-rarefaction* function in QIIME 2 by mink populations with weighted Unifrac distances with 20 iterations with mean ceiling at 10,000 sequences rarefaction.

The differential abundance analysis was conducted on the raw ASVs count, using the *DESeq2* package (Love et al., 2014), with a negative binomial Wald test to test significance between each group. Only phylotypes with a significance level (α) below 0.001 after false discovery rate (FDR) corrections were considered using the Benjamin–Hochberg method. All phylotypes were tested in contrast, meaning that differential abundance was done pairwise between each mink population. ASVs below the significance level and with a negative log2 fold change had thus their abundance significantly lower in the first group tested, and a positive log2 fold change indicated that the phylotype was significantly higher in the first group compared to the other group. I conducted this analysis to test differential abundance first, between captive and free-ranging populations within mink species and second between free-ranging populations between and within mink species.

4.3.6 Microsatellite markers genotyping, and analysis

A total of 94 hair and tissue samples were extracted from a larger dataset of samples from European and American mink over a ten-year period between 2000 and 2019 (unpublished data). All samples derived from the same population that the fecal samples originated from, but many from different individuals. Eighteen free-ranging American mink were sampled in Brittany (Côtes d'Armor), thirty American mink were sampled in the Pyrenees Atlantiques (Southwest region of France), as well as thirty individuals from the same fur farm in the southwest of France. Finally, ten captive European mink were sampled in captive settings at the FIEB breeding center and six free-ranging European mink were sampled in Navarra (Spain).

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN) from tissue and hair samples. Negative controls were also used. Multilocus genotypes were obtained by PCR amplification of 10 autosomal microsatellites (Fleming et al., 1999; Cabria et al., 2007). The forward primer of each locus was 5'-end labeled with a fluorescent dye. The following multiplex sets were designed: mix 1 (MLUT 25, MLUT 27, Mvis 099) and mix 2 (MLUT 04, MER009, Mvis075, Mvis072, MER41, MER022). PCR and genotyping steps were carried out following Pigneur et al. (2019). Length variation determination (alleles and genotypes) was performed using Genemapper 4.0 (Applied Biosystems). To construct consensus multilocus genotypes, an allele was only accepted if observed at least twice. I thus accepted heterozygous genotypes that were observed twice. A homozygote was accepted after three positive PCRs gave the same single allele.

The genetic structure of the mink populations was inferred using Bayesian clustering analysis with Structure 2.3 software (Pritchard et al., 2000). I ran 10 iterations for each K value from 1 to 10 using the admixture model. A total of 10^6 MCMC repetitions were performed after a

burn-in period of 20%. The results of the 10 iterations for each K value were summarized and averaged using the Clumpp method (Jakobsson & Rosenberg, 2007). The optimal number of clusters was investigated using the ΔK method (Evanno et al., 2005). For subsequent analyses, individuals were sorted according to their geographic origin (sorted into 5 main populations: Brittany, Nive basin, Navarra, Farm and Breeding Center, Figure 1). Mean allelic richness by locus (Ar), the expected (He) and observed (Ho) heterozygosity were calculated for each defined group using *diveRsity* (Keenan et al., 2013). An Euclidian distance matrix was constructed using GenAlEx 6.5 (Peakall and Smouse 2006), and a PERMANOVA model Adonis was conducted in a similar manner to β-diversity gut bacterial analysis with species and population. A UPGMA dendrogram was also constructed based with average linkage based on Fst values between mink populations.

4.4 Results

4.4.1 Microsatellite markers analysis

Overall, the three American mink populations had greater total allele count (A), percentage of heterozygous locus (%H), allelic richness (Ar), observed heterozygosity (Ho) and expected heterozygosity (He) than the European mink populations (Table 2). This suggests greater heterozygosity and genetic diversity in neutral markers for the American mink, and I observed even higher values for American mink in the fur farm compared to feral conspecifics. Bayesian clustering assignment recovered three distinct genetic clusters within our populations (Figure 2; Table S1); the European mink individuals form one cluster (K2), American mink from the farm and the Nive basin another (K1), and individuals from Brittany overlap on 2 clusters (K1 and K3; Figures 2& 3A). Only three American mink had admixture patterns between the two American mink clusters and belong to the fur farm population. Genetic distances between individuals' analysis through a PERMANOVA model indicated significantly greater distance between mink species than within, as well as according to mink population (Adonis: $F=7.6547$; $R^2=0.07206$; $p=0.0009$; $F=3.1927$; $R^2=0.09016$; $p=0.0089$, respectively). Finally, a dendrogram based on Fst distances between populations revealed that the mink population sampled had lower distance within species than between species (Figure 3A).

4.4.2 Comparison of α-diversity in gut bacterial

Samples of a mock community containing known concentrations of genomic DNA from 20 bacterial strains were sequenced. Nineteen of the twenty different strains originally included in the sample were detected. Therefore, our protocol allowed bacterial DNA detection and identification to the genus level as long as its concentration in the DNA extract was at least

2.8 pg/µl, and provided that the sequence was included in the reference database. Following the raw data processing, I obtained 1,947,964 sequences belonging to 3,036 distinct bacterial phylotypes (ASVs) after removal of negative control sequences, for 52 samples (other samples were removed during CSS normalization due to low sequencing depth).

Gut bacterial phylotypes richness did not significantly vary according to host species or host sex, when considering three richness measures (Table 2). However, both captive mink species tend to have lower bacterial phylogenetic diversity and lower Shannon indexes compared to conspecific free-ranging mink (χ^2=10.59, p-value=0.001137; and χ^2=2.9118, p-value=0.08793, respectively; Figure 4B). The Shannon index also significantly varied according to mink populations (χ^2=11.681, p-value=0.01989). When conducting a Dunn test for multiple comparisons with Benjamin & Hochberg correction for p-values, captivity seemed to have a strong negative impact on gut bacterial richness for both host species, especially compared to the American mink population from Brittany (Figure 4A&B).

4.4.3 Comparison of β-diversity of gut bacteria between groups

As expected in mustelid gastrointestinal tracts, all samples were dominated by the *Firmicutes* and *Proteobacteria* phyla, mostly belonging to the *Clostridiaceae* and *Peptostreptococcaceae* families (Figure 5; Compo et al., 2018; Bahl et al., 2017). The gut bacterial community composition of male and female mink for both species considered in the study (Adonis: F=0.314; R^2=0.0058; p=0.725) were not significantly different and explained around 0.5% of the variation. Thus, males and females were not treated separately in subsequent statistical analyses. Host species did not have a significant effect on gut community composition, as it explained 1.75% of the community variation (Adonis: F=0.938; R^2=0.0175; p=0.2827). However, 20.9% of gut bacterial composition variation was explained by mink belonging to the different populations in both weighted and unweighted Unifrac distances (Adonis: F=3.3127; R^2=0.20949; p=0.003996; and F=1.859; R^2=0.07478; p=0.005994, respectively, Figure 6). The variation seemed to be mainly explained by free-ranging or captive conditions (Figure S1). I did observe significantly greater distances between feral American mink in Brittany and other American mink groups, but no differences were detected between both captive and free-ranging European mink and American mink in Brittany (Figures 6&S1). A wide interindividual variation in gut bacterial composition was also observed in free-ranging European mink (Figure S1). Overall, feral American mink in Brittany and free-ranging European mink had lower β-diversity between them than any other mink populations (Figure 3B).

4.4.4 Differential bacterial abundance analysis

The assessment of the differential abundance of bacterial phylotypes using a negative binomial Wald test was conducted on the core microbiota of 391 phylotypes. From those, 141 phylotypes from nine phyla varied significantly among the mink populations with 82% belonging to *Firmicutes* and *Proteobacteria*. When comparing captive and free-ranging populations within mink species, feral American mink had phylotypes differentially abundant to captive conspecifics, from 100 to 65 ASVs, most of them decreasing (Table S2). Feral American mink had a ratio of 1.77 and 2.6, expressing more decreases than increases in taxa abundance in the natural environment compared to captive conspecifics. This decrease in taxon abundance between free-ranging and captive populations is higher in American than European mink (0.7). A large portion of those phylotypes belonged to the *Bacteroida* (families *Flavobacteriaceae, Muribacculaceae* and *Chitinobacteraceae*) and *Clostridia* (genera *Rhomboustia* mostly) classes (Table S2). However, when comparing free-ranging populations of both species, I observed more taxa abundance variation between the two populations of free-ranging American mink (64 taxa differentially aunbdant), than variation between American and European mink (53 taxa for the Nive basin and 42 taxa for Brittany). Feral American mink in Brittany had more phylotypes abundances in common with free-ranging native European mink than its conspecifics from the Nive basin (Table S2). Most of the abundance variation was attributed to reduction in ASVs belonging to the *Firmicutes* phylum (*Lactobacillus*, *Clostridium* genera and *Peptostreptococcaceae* family) and *Gammaproteobacteria* class between the two American mink populations.

4.5 Discussion

4.5.1 On the influence of human impacts on the mink gut microbiota

This study is the first to examine how the gut bacteria of riparian carnivores vary between related species with similar ecological niches in the context of farming, invasion, and conservation. Our results did not find any support for phylosymbiosis, as genetic relationships were not reflected in the composition of the gut microbiota (Figure 3). There was also a reduction in the richness of the bacterial community in captivity that surpassed any host species differences. A similar pattern was further observed in β-diversity measures. This trend has already been observed in other host taxa with a carnivorous diet (*Canidae*, McKenzie et al., 2017). It is currently well established that animals living in captivity experience a range of changes that can influence their gut bacteria, from diet change, veterinary care, specific and uniform environmental substrates, as well as reduced contact with conspecifics and other species. While most of the current literature compared free-ranging animals to individuals kept in zoos (Clayton et al., 2016; Borbón-García et al., 2017; Wasimuddin et al., 2017), the same trend is expected between feral and farmed mink.

I also observed differentially abundant taxa in free-ranging mink compared to captive conspecifics. In addition, bacterial loss was stronger in the invasive American mink than the native European species when comparing free-ranging populations to captive conspecifics. In this regard, feral American mink would have experienced less recolonization from gut bacteria in natural habitats than the European mink, when compared to their captive conspecifics. By nestedness and turnover of bacterial communities, feral American mink would have left a subset of captive gut microbes during the invasion process, potentially leading to pathogen loss. However, many successful invasions have occurred without any pathogen loss and further investigation on targeted bacteria would be required (Ansellem et al., 2017).

There are three potential ways that can explain a stronger pattern of differentiation in gut bacteria communities between feral to captive settings in the American mink compared to the European mink. First, the two species have very different conditions in captivity. Farmed American mink are held in individual and open-air elevated cages with minimal substrate and enrichment, whereas European mink are held in an enclosure with access to a pond, natural substrates and enrichment (branches, vegetation, mud). Moreover, the diet of the American mink is composed of processed fish and chicken, whereas the diet of the European mink consists of whole fish, chicken and mice. Those differences in captive conditions could explain the significant difference in the bacterial communities between wild and captive American mink, compared to the European mink.

Second, when considering free-ranging European mink in their natural habitat, they could be more likely to select specific gut bacterial taxa because of their shared coevolutionary history with the environmental microbes in western France (Bankers et al., 2021). On the other hand, the invasive American mink may lack host-microbes coevolutionary history with native bacteria and would thus be less likely to retain newly acquired microbes when becoming feral. It is worth noting that the estimated divergence time between the two mink species is 8.28 million years ago (Hedges et al., 2006), and further research with other native mustelids such as the European polecat (*Mustela putorius*), that diverged more recently from the European mink could give more insight into gut bacteria colonization from wild to captive settings.

The third explanation relies on the evolutionary history of the American mink itself. The domestication process of this species started in the 1860s in Canada (Morris et al., 2020), as humans selected animals with dense, soft and shiny fur, as well as increased fertility to maximize their revenue. Docility, also termed confidence towards humans, was another behavioural trait that many European breeders favoured to facilitate daily handling and improved welfare (Thirstrup et al., 2019). Thus, genetic and phenotypic differences have already been observed between free-ranging and farmed American mink, including smaller

brain size, longer transit time and increased nitrogen metabolism in farmed animals (Morris et al., 2020; Bowman et al., 2017; Gugolek et al., 2012; Kruska, 1996). This explanation seems consistent with the high genetic diversity in mink from the fur farm observed in this study compared to free-ranging American mink populations. There is increasing evidence of the important interactions between the gut microbiota and the gut-brain axis in many species, including farm animals (Collins et al., 2012; Kraimi et al., 2019). It would be likely that artificial selection might have impacted the overall gut microbiota composition of the American mink through morphological and physiological variation, and thus changes in the gut-brain-axis, compared to the European mink that has not experienced domestication. The adverse effects of domestication on gut bacteria have already been observed in other mammals (Prabhu et al., 2020). However, to confirm either of both explanations on those exploratory results, further investigation with larger sampling size should be conducted.

4.5.3 No phylosymbiosis signal observed in mink

In general, our results did not support the phylosymbiosis hypothesis, and it was observed that the host environment had a strong influence on the mink gut microbiota. First, neither gut bacterial α- or β-diversity varied according to host species. Second, the feral American mink groups were more distinct from one another than with the free-ranging European mink, despite belonging to the same species. Furthermore, feral American mink in the Nive basin had less similarly abundant bacterial taxa in common with free-ranging European mink than feral American mink in Brittany. The absence of a phylosymbiosis signal is consistent with the fact that despite not being the most diverse population genetically, the invasive American mink from Brittany are the most genetically differentiated from the other American mink populations, being composed of at least three different genetic clusters. Three genetic pools have already been documented in this long-established population due to accidental releases over multiple introduction events, fostering diversity but also genetic drift (Bifolchi et al., 2010).

Similar to formation of a distinct population through genetic drift within farms, an analogous concept termed ecological drift might have occurred in gut microbes between mink populations, in relation with the ecology of the host (Kohl, 2020). These shifts in bacterial composition between free-ranging mink species could be explained by variation in prey availability due to habitat differences between the areas sampled. Studies in other parts of Europe showed that the American mink has a plastic diet (Maran et al., 1998; Zalewski & Bartoszewicz, 2012; Chibowski et al., 2019). When found in agricultural landscapes, the mink tend to feed on ground-dwelling small mammals, such as *Microtus* sp that are highly abundant in rural habitats (Krawczyk et al., 2013). Considering the variation in landscapes in our study (Figure 1), the Côtes d'Armor area in Brittany is more subject to anthropogenic activities

compared to the Nive watershed in the Southwest. The latter is mainly composed of forests (48%) and meadows (30%; MNHN, 2015), while the Côtes d'Armor landscape was dominated in 2015 by agricultural areas (56%), then forests (21%) and very few meadows (9%; DRAAF Bretagne, 2021). A study conducted in northeastern Spain observed that the free-ranging American mink mostly predated on crayfish and this might be reflective of the mink diet in the Nive watershed (Melero et al., 2008). Alternatively, it is possible that feral American mink in Brittany have a similar diet to the mink from agricultural landscapes in Poland, preying on the available ground-dwelling rodents (Krawczyk et al., 2013). This difference in diet related to landscape variation between the two American mink populations could thus be reflected in the different composition of the gut microbial communities (Reese & Dunn, 2018; San Juan et al., 2020).

Regarding the free-ranging European mink habitat, the land uses of Navarra in 2015 was primarily agricultural areas (34.8%) and forests (28.2%), followed by meadows (15.7%; Vicente et al., 2005). The greatest proportion of agricultural lands in both Navarra and Brittany could thus indicate similar prey availability compared to the Nive watershed. Palazon et al. (2004) observed that the European mink diet in Navarra and La Rioja was predominantly composed of small mammals and fish, thus supporting the hypothesis that gut microbial composition of both mink species according to prey availability based on land occupation. To date, little is known about the diet of each mink species where our samples originated, but further work on their diet and gut bacteria, as well as prey surveys in mink territory could validate this hypothesis.

In conclusion, this study provides insight into the relationship between the gut bacteria of invasive and native carnivorous mammal hosts, with no observable signals of phylosymbiosis due to the strong influence of the environment and diet of the host on its associated microbes. Studying gut microbiota differences between mink farms in multiple countries, as well as individuals in their native habitat could also give more insight into the effects of domestication on microbe-host relationships.

4.6 References

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4.7 Tables and Figures

	American mink			European mink	
	Brittany	Nive basin	Farm	Spain	Breeding Center
N	17.6	27.9	25.4	5.8	9.7
A	66	80	97	18	22
%H	41.69	49.8	59.7	11.95	14.8
Ar	3.92	4.15	4.83	1.63	2.03
Ho	0.39	0.48	0.61	0.23	0.49
He	0.66	0.7	0.77	0.19	0.35
Fis	0.4043	0.3142	0.2144	-0.1869	-0.3894
Fis_Low	0.2886	0.2412	0.139	-0.468	-0.5148
Fis_High	0.5032	0.3935	0.2868	0.0324	-0.2669

Table 1. Sample size without missing data (N), total allele count (A), percentage of heterozygous locus (%H), allelic richness (Ar), observed heterozygosity (Ho), expected heterozygosity (He), and inbreeding coefficient (Fis) for each mink population.

	Chao1		Shannon index		Faith's PD	
	χ2	p-value	χ2	p-value	χ2	p-value
Host species	0.472	0.492	1.634	0.201	0.169	0.680
Host sex	1.651	0.198	0.178	0.673	1.946	0.163
Mink population	3.527	0.474	*11.681*	*0.019*	5.829	0.212
Host environment	1.680	0.195	*10.59*	*0.001*	2.919	0.088

Table 2. Kruskal-Wallis chi-squared and p-values from tests for each alpha diversity metrics according to each variable investigated. Italicized values meet the standard cut-off for statistical significancy.

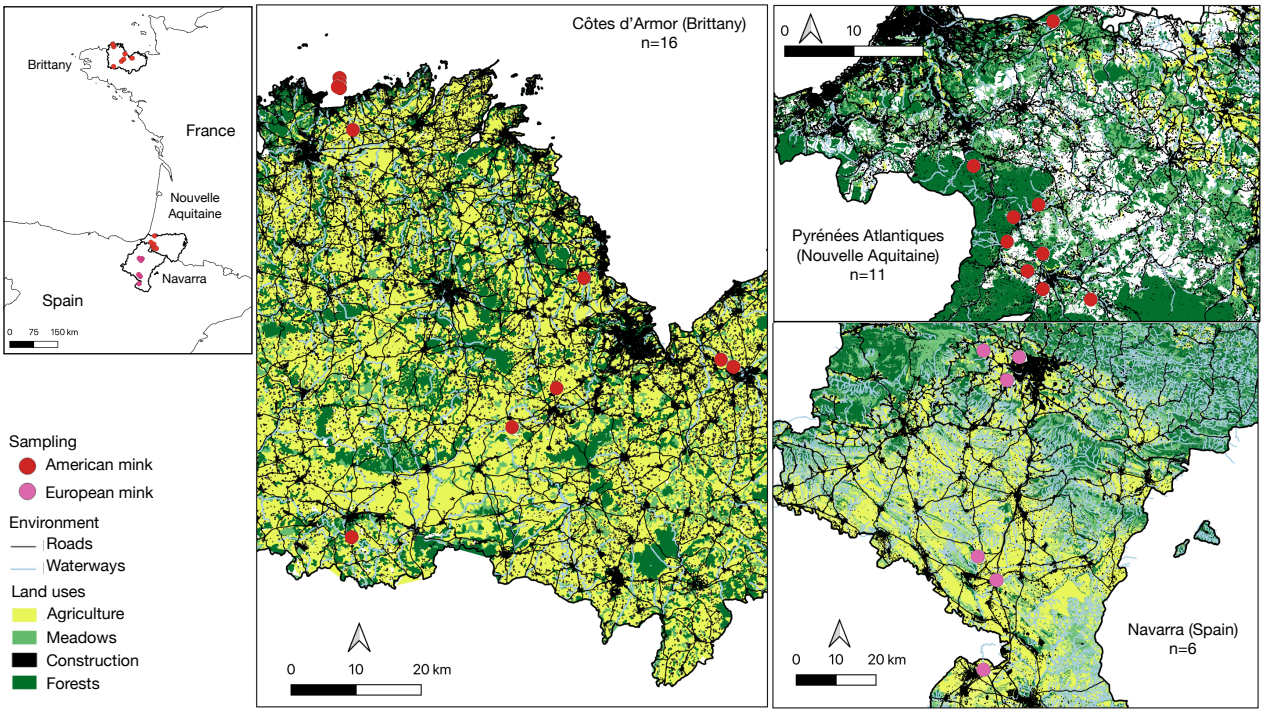

Figure 1. Map of free-ranging mink sampling sites with land uses.

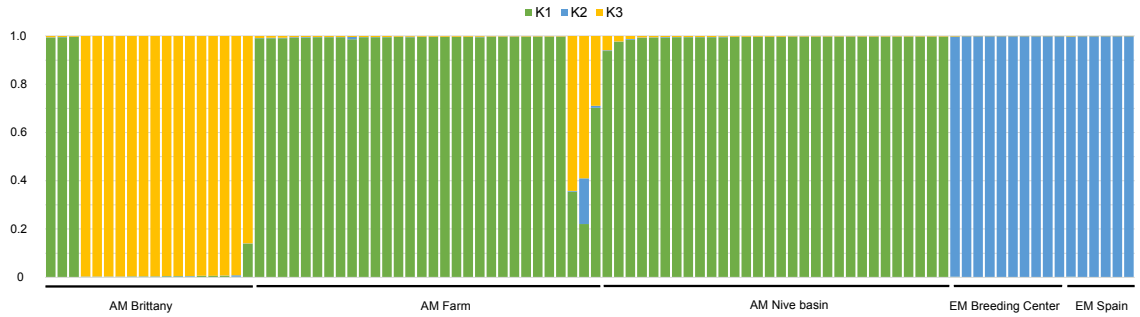

Figure 2. Individual assignment for each mink sampled according to Bayesian clustering following Evanno Best K method (K=3) based on microsatellite data.

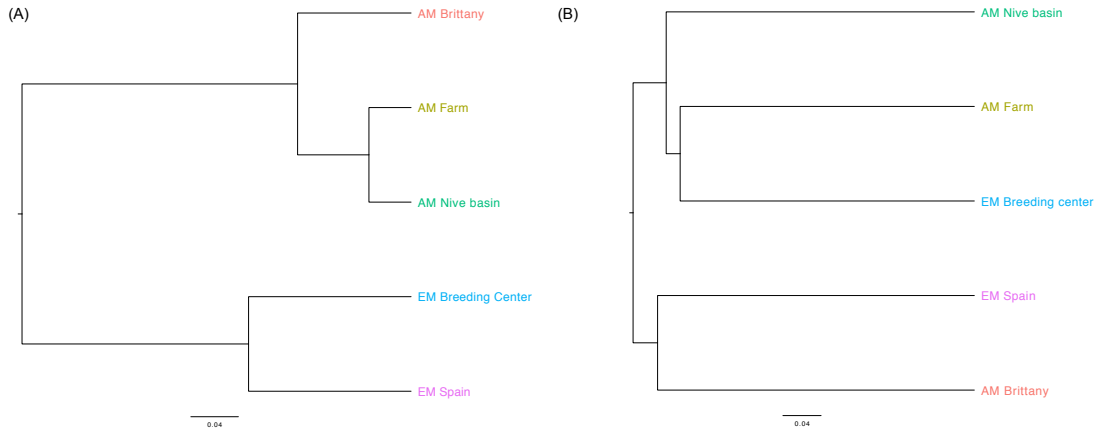

Figure 3. (A) UPGMA dendrogram constructed with Fst values from microsatellite data between mink population sampled, and (B) from weighted Unifrac distance matrix based on mean ceiling of each sample grouped by mink populations for gut microbial β-diversity.

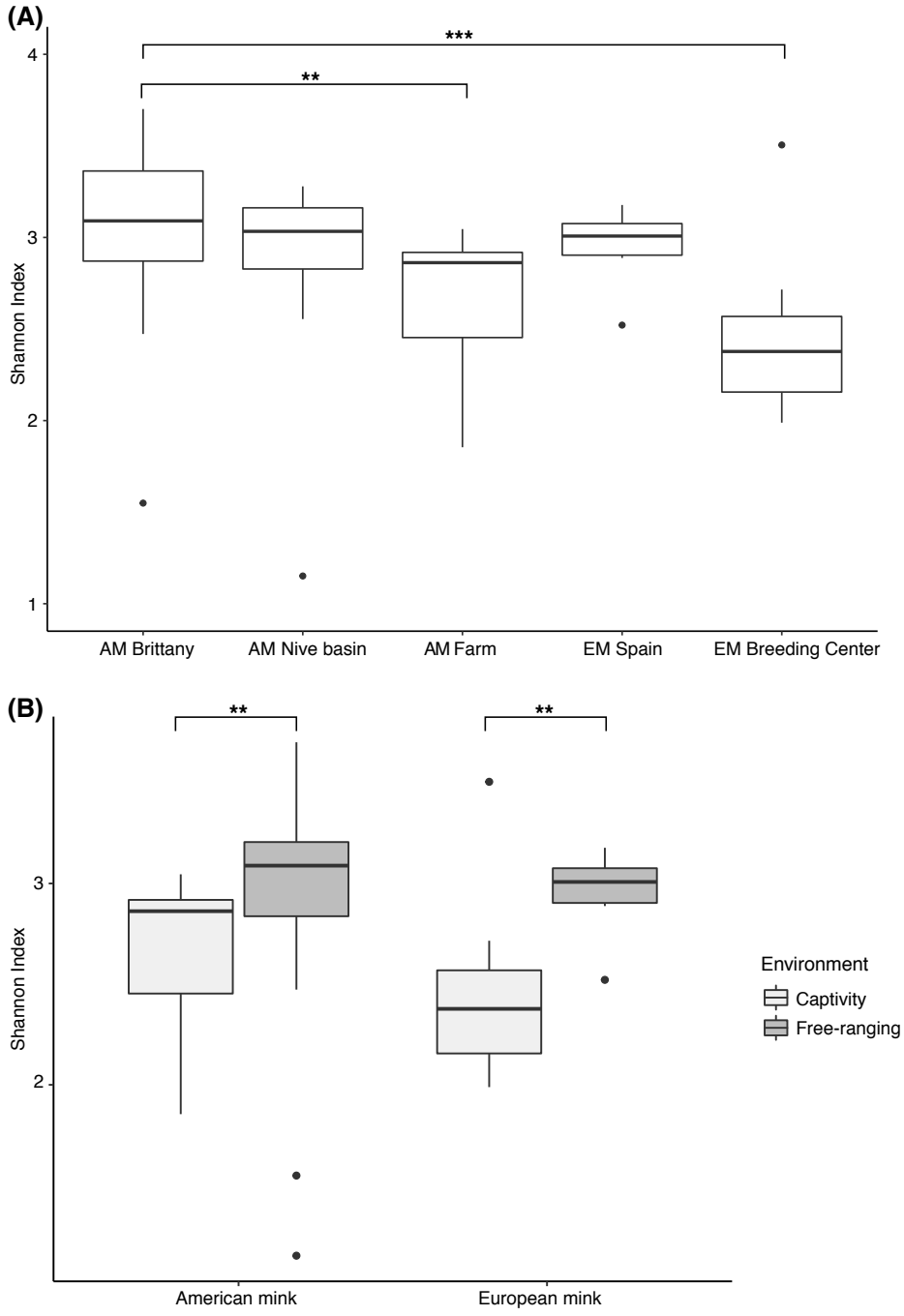

Figure 4. Boxplots representing Shannon Index variation of the gut microbiota depending on (A) host's environmental group, ** represents the p-value meeting the standard cutoff p<0.01 and *** p<0.001 from by Dunn test of Kruskal-Wallis multiple comparisons with Benjamini & Hochberg correction, and depending on (B) host's environment for both mink species.

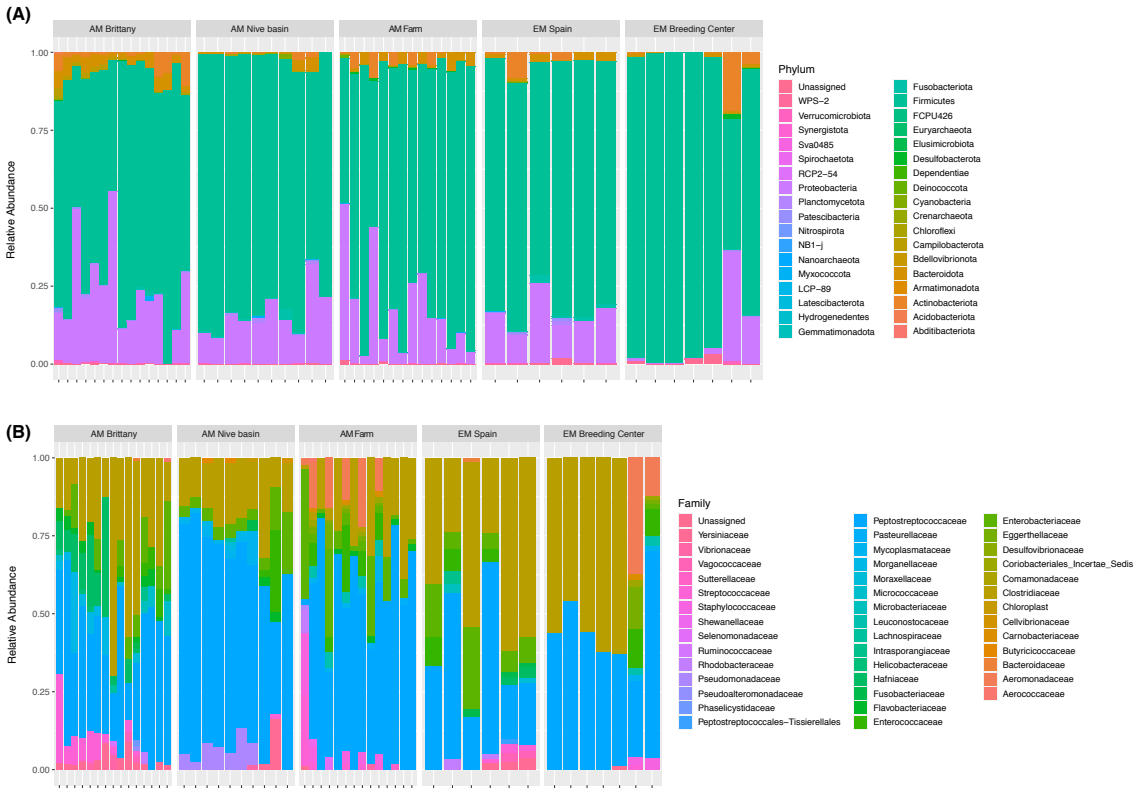

Figure 5. Compared relative abundance of bacterial taxa for each group of mink in the study (taxa showing less than 0.1% of relative abundance were not included). In each group, samples are sorted by individual. Stacked barplot showing the relative abundance at the (A) phylum and (B) family levels for gut bacteria.

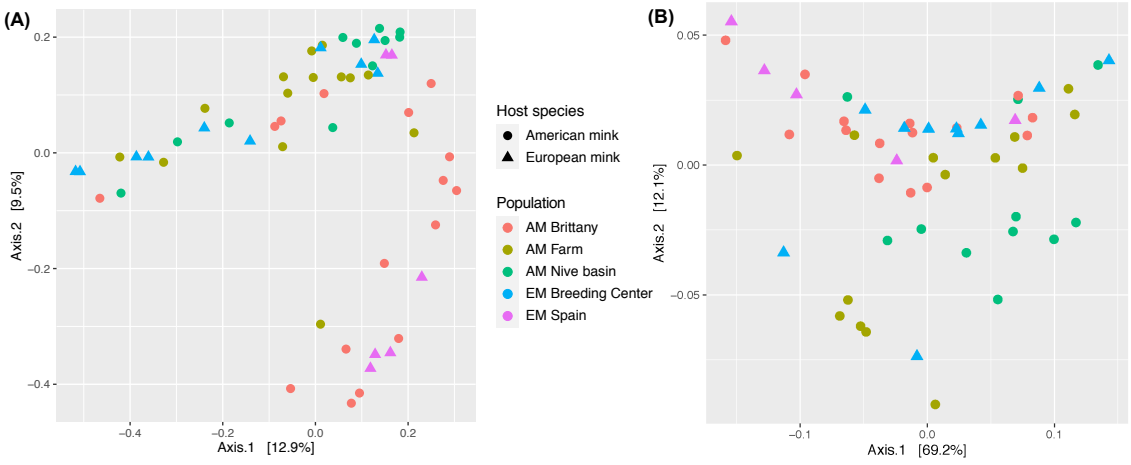

Figure 6. PCoA on (A) unweighted and (B) weighted Unifrac metric between samples. Unifrac metric calculated between samples for all gut bacterial taxa. Colors represent host population and shape the host species.

4.8 Supplementary materials

K	Iterations	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-3911.3500	0.8086	NA	NA	NA
2	10	-3155.3300	10.3962	756.02	373.98	35.972903
3	**10**	**-2773.2900**	**1.7760**	**382.04**	**308.49**	**173.694992**
4	10	-2699.7400	13.3695	73.55	46.19	3.454887
5	10	-2672.3800	16.9493	27.36	9.150	0.539847
6	10	-2635.8700	35.7728	36.51	4.58	0.128030
7	10	-2603.9400	40.6407	31.93	7.21	0.177409
8	10	-2579.2200	24.9257	24.72	6.57	0.263584
9	10	-2561.0700	45.6596	18.15	32.07	0.702372
10	10	-2574.9900	42.3949	-13.92	NA	NA

Table S1. Evanno method output values for Best K from Structure results.

p<0.001 DESEQ2 ASV	Environmental groups			Wild VS Captive populations						Wild American mink VS European mink populations									
	AM Brittany_AM Farm			AM South West_AM Farm			EM Spain_EM Breeding Cen			AM Brittany_EM Spain			AM South West_EM Spain			AM South West_AM Brittany		
ASV taxonomy	Decrease	Increase	Total	Decrease	Increase	Total	Decrease	Increase	Total	Decrease	Increase	Total	Decrease	Increase	Total	Decrease	Increase	Total
p__Actinobacteriota	*6*	*2*	*8*	*7*	*2*	*9*	*6*	*1*	*7*	*1*	*3*	*4*	*1*	*2*	*3*			
c__Actinobacteria	1	2	3	1	2	3		1	1	1	3	4	1	2	3			
f__Micrococcaceae		2	2		2	2					2	2		2	2			
c__Coriobacteriia	4		4	5		5	5		5									
f__Coriobacteriales_Incertae_Sedis				1		1	1		1									
f__Eggerthellaceae	4		4	4		4	4		4									
c__Thermoleophilia	1		1	1		1	1		1									
p__Bacteroidota	*5*	*1*	*6*	*6*		*6*		*2*	*2*	*1*		*1*	*3*		*3*	*2*		*2*
c__Bacteroidia	5	1	6	6		6		2	2	1		1	3		3	2		2
f__Bacteroidaceae		1	1	1		1		1	1				2		2	2		2
f__Chitinophagaceae	1		1	1		1												
f__Flavobacteriaceae	2		2	2		2		1	1	1		1	1		1			
f__Muribaculaceae	2		2	2		2												
p__Campilobacterota											*2*	*2*						
g__Helicobacter											2	2						
p__Cyanobacteria	*1*		*1*		*1*	*1*	*1*		*1*							*1*		*1*
p__Desulfobacterota	*1*		*1*	*1*		*1*	*1*		*1*									
g__Desulfovibrio	1		1	1		1	1		1									
p__Firmicutes	*31*	*29*	*60*	*22*	*8*	*30*	*14*	*20*	*34*	*5*	*21*	*26*	*16*	*15*	*31*	*31*	*18*	*49*
c__Bacilli	15	3	18	16	1	17	10	4	14	4	2	6	7	2	9	7	3	10
f__Enterococcaceae				1		1							1		1	1		1
f__Erysipelotrichaceae	1		1											1	1		1	1
f__Gemellaceae	1		1	1		1	1		1									
f__Lactobacillaceae		1	1	2		2		1	1	1		1	2		2	3		3
f__Leuconostocaceae				1		1	1		1	1		1				1		1
f__Mycoplasmataceae	3		3	2		2	1		1				1		1		1	1
f__Planococcaceae	1		1	1		1				1		1						
f__Staphylococcaceae	2		2	2		2	2		2									
f__Streptococcaceae	7	1	8	6	1	7	5	2	7	3		3	2		2	1	1	2
g__Lactococcus	1	1	2	1	1	2	1	1	2	1		1	1		1			
g__Streptococcus	6		6	5		5	4	1	5	2		2	1		1	1	1	2
f__Vagococcaceae		1	1					1	1				1		1	1		1
c__Clostridia	16	26	42	6	6	12	4	16	20	1	19	20	9	12	21	24	14	38
f__Butyricicoccaceae		1	1		1	1		1	1									
f__Clostridiaceae	4	14	18	5	4	9	3	8	11		12	12	6	5	11	13	3	16
f__Lachnospiraceae	1	1	2		1	1					1	1		2	2	1	2	3
f__Peptostreptococcaceae	11	8	19	1		1	1	6	7	1	5	6	2	5	7	8	9	17
g__Paraclostridium								2	2									
g__Peptostreptococcus		2	2					2	2				1		1	2		2
g__Romboutsia	8	3	11	1		1		1	2	1	3	4		4	4	3	6	9
g__Terrisporobacter	2	3	5					1	1		2	2	1	1	2	3	2	5
g__Unassigned	1		1														1	1
f__Peptostreptococcales-Tissierellales		1	1					1	1		1	1	1		1	1		1
f__Ruminococcaceae		1	1								1	1				1		1
c__Negativicutes				1	1									1	1		1	1
g__Megamonas				1	1									1	1		1	1
p__Fusobacteriota	1	1	2				1	1					1	1	2	1	1	2
g__Fusobacterium	1	1	2				1	1					1	1	2	1	1	2
p__Myxococcota		1	1	1		1												
g__Phaselicystis		1	1	1		1												
p__Proteobacteria	*10*	*11*	*21*	*7*		*18*	*7*	*12*	*19*	*1*	*8*	*9*	*4*	*7*	*11*	*7*	*3*	*10*
c__Alphaproteobacteria	3		3				2	1	3	1		1	1		1			
f__Beijerinckiaceae	1		1				1		1									
f__Rhodobacteraceae	2		2				1	1	2	1		1	1		1			
c__Gammaproteobacteria	7	11	18	7	7	15	5	11	16		8	8	3	7	10	7	3	10
f__Alcaligenaceae	1		1	1		1												
f__Comamonadaceae								1	1									
f__Enterobacteriaceae		2	2	1	1	2	2	4	6		2	2	1	2	3	1		1
f__Hafniaceae		2	2								1	1				1		1
f__Moraxellaceae		2	2	1	1	2		2	2				2		2	2		2
f__Morganellaceae		2	2	1	2	3		2	2		2	2		1	1	1		1
f__Pasteurellaceae	2		2	2		2	1		1									
f__Pseudoalteromonadaceae		1	1					1	1		1	1				1		1
f__Pseudomonadaceae	1		1	1	3	4	1	1	1					2	2		2	2
f__Shewanellaceae	1		1	1		1												
f__Sutterellaceae	1		1	1		1												
f__Vibrionaceae	1	1	2								1	1		1	1	1	1	2
f__Yersiniaceae		1	1								1	1		1	1			
Total	**55**	**45**	**100**	**47**	**18**	**65**	**28**	**36**	**64**	**8**	**34**	**42**	**25**	**28**	**53**	**41**	**23**	**64**

Table S2. Results from the DESEQ2 differential abundance analysis between mink populations. Each number represents the number of phylotype from each taxon, organized according to their taxonomy, either significantly lower (decrease) or higher (increase) in abundance in the first population compared to another population documented in each column.

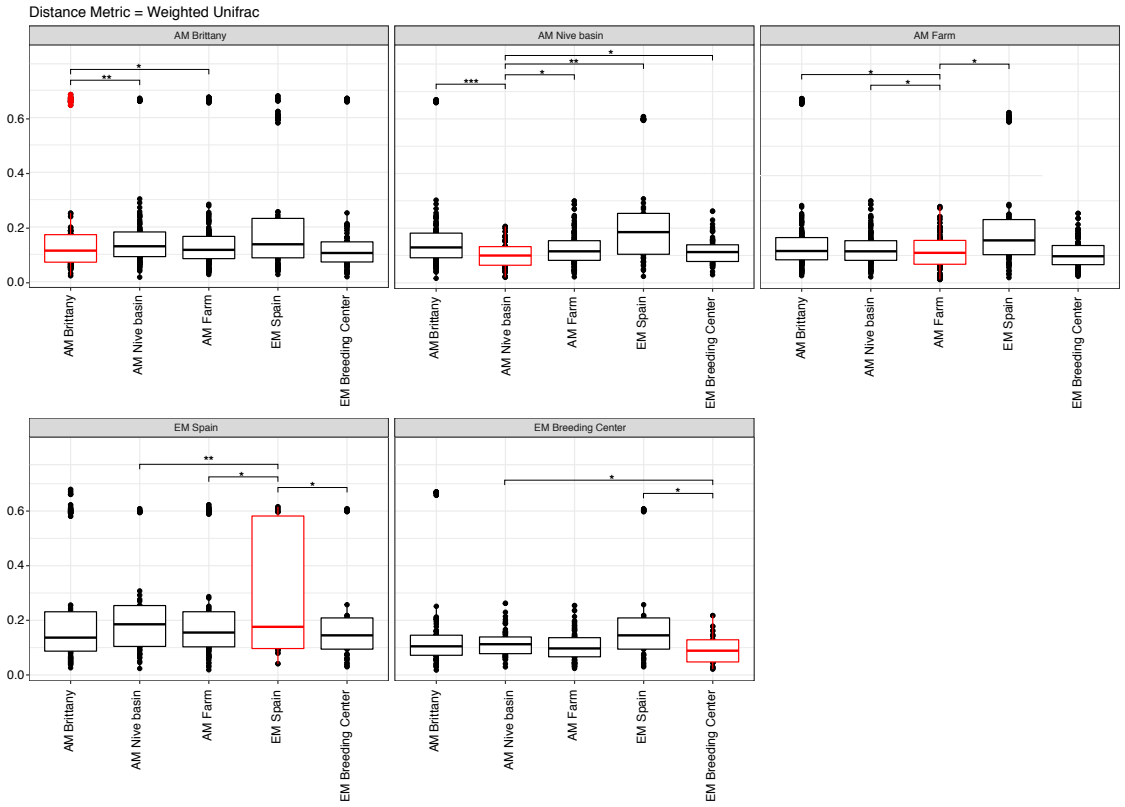

Figure S1. Weighted Unifrac distances comparison boxplots of all samples between and within populations. The boxplot in red represents the beta dispersion within the group and in black of the said red group between all the other groups. *** represents the p-value meeting the standard cutoff p<0.001, ** p<0.01, and * p<0.05.

Chapter 5. Conservation genetics and gut microbial communities' variability of the critically endangered European mink *Mustela lutreola*: Implications for captive breeding programs

5.1 Summary

Host's fitness can be affected by its genotype and gut microbiota, defined as the microbes living in the host's intestinal tract. This study explored how the genetic diversity of the host influences its bacterial communities in the context of captive breeding programs, for the

critically endangered European mink (*Mustela lutreola*). As stated by the ecosystem on a leash model, loss of host genetic diversity may lead to changes in immunomodulation and will therefore induce modifications of the gut microbiota. I investigated variation in the gut bacteria through 16S rRNA metabarcoding, related to the genetic diversity of European mink held in captivity in two breeding centers representing separate breeding stocks originating from the western and eastern populations. The genetic diversity of the host was assessed through diversity analysis of the adaptive MHC class I and II genes as well as neutral microsatellite markers. Results indicate lower diversity in neutral and MHC class I genes for the western population, and the opposite for MHC class II. A lower MHC class II gene variability led to an increase in microbial phylogenetic diversity and in abundance depending on the presence of specific MHC-II motifs. Those results seem to be linked to management practices that differs between the two programs, especially the number of generations in captivity. Long term *ex situ* conservation practices can thus modulate gut microbial communities, that might potentially have consequences on the survival of reintroduced animals. I suggest strategies to foster genetic diversity in captive breeding program to mitigate the effects of genetic drift on those small, isolated populations.

RESEARCH ARTICLE

Conservation Genetics and Gut Microbial Communities' Variability of the Critically Endangered European Mink *Mustela Lutreola*: Implications for Captive Breeding Programs

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5.2 Introduction

More than 5,800 animal species to date are endangered, as the Earth experiences a mass extinction event (Ceballos et al., 2015). Intrinsic drivers of extinction, such as genetic factors, play a key role for population viability, especially when species are reduced to small, isolated populations that can be negatively affected by genetic load (Hedrick, 2001). In this scenario, finding a suitable mate is challenging and reproduction with related individuals can occur,

leading to inbreeding depression. Inbreeding has largely been documented in small populations in the wild (Hedrick, 2001; reviewed in Spurgin & Gage, 2019), impacting population fitness through the fixation of detrimental alleles. An increase of detrimental alleles in endangered species increases their susceptibility to extrinsic ecological drivers of extinction (Frankham, 2005). One *ex situ* conservation tool used to mitigate the decrease of genetic diversity in endangered species are Captive Breeding Programs (CBPs). Captive populations of endangered species have the difficult goal of ensuring the survival of stable, self-sustaining populations for later reintroduction into the native habitat (Mallinson, 1995). A key challenge of CBPs is to maintain genetic diversity and avoid inbreeding depression with a small number of founders (Bouman, 1977; Ralls et al., 1979).

The majority of captive breeding schemes rely on studbooks that document pedigree information within the CBPs. While studbooks can be useful to minimize inbreeding effects (Pelletier et al., 2009; Witzenberger & Hochkirch, 2011), information from pedigrees can be flawed in some captive populations (Bowling et al., 2003; Marshall et al., 1999; Signer et al., 1994). Molecular genetic analyses can provide more insights into the relationships within captive populations and their genetic structure. Recently, genetic studies of endangered species have increased, using highly variable loci non-coding for fitness traits such as microsatellite markers (Witzenberger & Hochkirch, 2011). Microsatellites are known to be highly informative as a tool to measure neutral genetic variation, and generally represent the extent and pattern of molecular variation within a population (Selkoe & Toonen, 2006). However, both empirical and simulated data indicate that patterns of variation and divergence in adaptive traits are not always associated with concomitant variation in neutral markers (Hedrick, 2001; Larson, 2012; Reed & Frankham, 2001), and some conservation biologists advocate for genetic diversity analysis for adaptive variation in CBPs (Hughes, 1991; Sommer, 2005). One targeted adaptive region is the Major Histocompatibility Complex (MHC) because its genes play a crucial role in the adaptive immune system. Historical events such as bottlenecks and founder effects, but also constraints of the mating system, such as limited sexual selection in CBPs (Schulte-Hostedde and Mastromonaco 2015), can be reflected in low numbers of MHC alleles (Schad et al., 2004; Hapke et al., 2004). However, in some free-ranging populations, genetic variation at the MHC might persist due to balancing selection, through rare allele fitness advantage, despite low levels of variability shown by neutral markers (Jarvi et al., 2004; Rico et al., 2016). These studies support the difficulty of using neutral markers as surrogates for variation in fitness-related loci.

MHC genes are considered one of the most diverse loci in jawed vertebrates and good candidates for genetic diversity analysis in endangered species (Hughes, 1991). They have a crucial role in adaptive immunity, by encoding proteins that bind peptide antigens and present them at the cell surface to lymphocytes for their activation (Ujvari & Belov, 2011).

MHC-I molecules are known to act at the intracellular level, while MHC-II molecules target extracellular non-self-recognition (Ost & Round, 2018). High genetic diversity in these loci could allow targeting numerous combinations of gut microbes (Ost & Round, 2018), reflected in variable immunity or tolerance among individuals through rare allele and heterozygous advantage in balancing selection.

Within this context, Foster et al. (2017) proposed a theoretical framework known as the ecosystem on a leash model, which posits that the host is under strong selection to evolve mechanisms to keep the microbiota under control. The presence of a genetically diverse microbiota leads to the dominance of the fastest growing microbes instead of the microbes that are most beneficial to the host (Foster et al., 2017). The targeting of microbial taxa to either limit their proliferation could thus be beneficial to the host, through its adaptive immune response. Bolnick et al. (2014) examined the role of MHC-II motifs (amino acid sequences) in gut microbial community variation in sticklebacks (*Gasterosteus aculeatus*) and found that common MHC motifs were linked to increases in microbial abundance and diversity, and rare motifs had the opposite impacts. Similarly, the microbiota was less phylogenetically diverse in individuals with high MHC-II diversity in the plumage of blue petrels (*Halobaena caerulea*, Leclaire et al., 2018), the gut of laboratory mouse strains (BALB/c, Khan et al., 2019), and the fur microbiota of fur seals (*Arctocephalus gazella*, Grosser et al., 2019). However, no study to date has investigated the MHC-gut microbiota relationships in endangered species under CBPs. I therefore hypothesize that less host control, expressed by more genetically diverse gut microbes, should happen in individuals with reduced genetic diversity in both neutral and adaptive markers. To test this hypothesis, I investigated the genetic diversity and gut microbial community assemblages in the critically endangered European mink (*Mustela lutreola*).

The European mink (E-mink) is a semi-aquatic carnivore from the *Mustelidae* family. Once widespread throughout Europe, it was evaluated as "critically endangered" in 2011 (Maran et al., 2016). There have been drastic declines in population and range, historically due to overexploitation and nowadays notably driven by habitat loss, degradation and fragmentation, road collisions, and the impacts of the alien American mink (*Mustela vison*). E-mink populations are now restricted to enclaves in western France and northern Spain, referred as the western population, while the eastern population is present in the delta of the Danube in Romania, Ukraine and Russia (Maran et al., 2016), the latter being the focal origin of a captive breeding effort in Estonia, with successful reintroduced populations on Hiiumaa Island.

Two major studies have documented the genetic diversity of the free-ranging E-mink populations (Michaux et al., 2005; Cabria et al., 2015). The western population had a much

lower microsatellite genetic diversity and allelic richness compared to the eastern population. The authors concluded that the western free-ranging population reached a recent bottleneck, and potentially inbreeding depression due to geographic isolation. However, no proof of fitness reduction in this population through inbreeding has been reported as of yet (Carbonell et al., 2015). The antigen-binding site, encoded by exon 2 of the DRB MHC class II gene, was also investigated in the eastern captive population by Becker et al. (2009). They detected nine alleles within the 20 individuals investigated, estimating low to moderate variability when comparing to other endangered species in similar situations to the E-mink. However, no comparison is yet available for the captive western population.

Both populations are currently in CBP's. The eastern breeding stock is only composed of captive-born individuals for over thirty generations (Maran, pers. comm., 2021) and is managed under an EAZA Ex situ Program (EEP). On the other end, the western breeding stock managed by the Fundación para la Investigación en Etología y Biodiversidad (FIEB) originates from the free-ranging western population captures in Spain within the last seven years (i.e. seven generations), and wild-born individuals from Spain are still being introduced as founders to this date. Those populations are considered as two distinct stocks and are bred separately, although few cross breeds are currently being conducted.

Both captive E-mink populations therefore offer a range of variation in neutral and adaptive genetic diversity. Due to extreme population variation over time and the emergence of small and isolated populations, the E-mink provides a unique framework to study the relationship between host genetics and gut microbial communities. Following the ecosystem on a leash model, the aims of this study were to (i) characterize the genetic diversity in the two captive E-mink populations with neutral and adaptive genetic markers as well as their gut microbial communities, (ii) examine the relationship between gut microbial diversity and genetic diversity, and (iii) investigate if gut microbial community structure and composition is linked to specific MHC motifs.

5.3 Methods

5.3.1 Sample collection and DNA extraction

Samples were collected from captive sexually mature mink from both populations in 2020. Fourteen E-mink were sampled in the EEP conservation breeding center at Zoodyssée in France (representing the eastern population), and ten E-mink were sampled in captive settings at the FIEB breeding center in Spain (representing the western population) Table S1]. Two mink sampled in the Spanish breeding center were wild-born individuals but spent at least a year in captivity before sampling. One individual sampled in the Spanish breeding

center was the result of a crossbreed between western and eastern mink. For MHC and microsatellite markers analysis, hair samples were collected using sterilized tweezers from each animal during a routine procedure. For the gut microbiota, fresh fecal samples were collected in the enclosure of each animal separately using sterilized tools and kept in 96% ethanol tubes at 4°C until further processing. As the E-mink's diet in captivity varies by day, samples were collected at four occasions depending on the item fed to the animal the previous day. The diet of the E-mink from both breeding centers relied on 3 types of food: trout, mice and chicken.

DNA from hair samples were extracted using the DNeasy Blood and Tissue Kit from Qiagen using the manufacturer's protocol. DNA extractions from the fecal samples collected were conducted in duplicates using the QiaAmp Mini Kit with Inhibitex (Qiagen, Germany) following the manufacturer's instructions. Two blank extractions were made to control for contamination during the extraction process. A mock community sample (HM-783D, BEI resources) containing genomic DNA from 20 bacterial strains, at concentrations ranging from 0.6 to 1400 pg/µl, was also added in each library to confirm the reliability of our method.

5.3.2 Microsatellite analysis

Multilocus genotypes were obtained by PCR amplification of 16 autosomal microsatellites (Fleming et al., 1999; Cabria et al., 2007). The forward primer of each locus was 5'-end labeled with a fluorescent dye. The following three multiplex sets were designed: mix 1 (Mvi 114, MLUT 25, MLUT 27, Mvis 099, Mvi 4001), mix 2 (Mvi 087, MLUT 32, MLUT 35, Mvis022, Mvi 1341) and mix 3 (MLUT 04, MER009, Mvis075, Mvis072, MER41, MER022). PCR and genotyping steps were carried out following Pigneur et al. (2019). Length variation determination (alleles and genotypes) was performed using Genemapper 4.0 (Applied Biosystems). To construct consensus multilocus genotypes, an allele was only accepted if observed at least twice. I thus accepted heterozygous genotypes that were observed twice. A homozygote was accepted after three positive PCRs gave the same single allele.

The genetic structure of both sampled captive breeding centers was inferred using Bayesian clustering analysis with Structure 2.3 software (Pritchard et al., 2000). I ran 10 iterations for each K value from 1 to 5 using the admixture model. A total of 10^6 MCMC repetitions were performed after a burn-in period of 20%. The results of the 5 iterations for each K value were summarized and averaged using the Clumpp method (Jakobsson & Rosenberg, 2007). The optimal number of clusters was investigated using the ΔK method (Evanno et al., 2005). F-statistics (pairwise F_{ST}, D_{Jost} and F_{is}), allelic richness (Ar), the expected (He) and observed (Ho) heterozygosity, as well as inbreeding coefficient (F) and multilocus heterozygosity (MLH) were calculated for each defined group/individual in R version 3.5.2 (R Development Core

Team, 2008). A R_{ST} genetic distance estimation matrix between individuals was generated using GenAlex 6.5 (Peakall & Smouse, 2006).

5.3.3 Amplification, sequencing and analysis of MHC genes

The fragment of DRB gene from exon 2 of MHC class II (Beta 1, 231 bp) was targeted using the primers designed by Becker et al. (2009) for *Mustela lutreola* and a fragment from exon 2 (alpha 1) from MHC class I, using the primers Meme-MHC-Iex2F and PpLAa1L250 designed by Sin et al. (2012) for mustelids. PCRs were carried out in 25 µl volumes containing 0.9 µl of primer mix, 5 µl of GoTaq reaction buffer (Promega), 2 µl of $MgCl_2$,0.04 µl of BSA, 0.8 µl of dNTPs, 0.125 µl of GoTaq G2 DNA polymerase (Promega, France) and 3 µl of DNA. The specific protocol was used for PCR: annealing with touchdown protocol from 65°C to 56°C for 30 s. Amplified DNA in duplicates were pooled after quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific Inc., Austria). The library preparation and sequencing were performed by Novogene (UK). Using their designated library protocol, 2×250 bp paired-end sequencing with a depth of 50,000 reads/sample for MHC genotyping and was completed using an Illumina NovaSeq platform (Illumina Biotechnology Co., Novogene, UK).

To analyze MHC-I and MHC-II amplicon sequences, I used the three-step pipeline AmpliSAS (Sebastian et al., 2015). Low-quality sequences with Phred scores lower than 20 were removed and clustering was conducted using the default parameters for Illumina sequences. Already identified alleles of MHC-II DRB for E-mink were extracted from NCBI (Becker et al., 2009), as well as sequences from related species (*Mustela putorius* and *Mustela itatsi*) for MHC-I exon 2. If NCBI blast reveled 100% of sequence identify between the discovered alleles in this study and already identified one, their name was replaced by the accession number of these sequences. For the subsequent analysis, I focused on the amino acid translated sequences (referred as MHC motifs) as they are in direct contact with bacteria. I measured motif richness as the number of sequences per individual for each locus. I calculated functional distances between individuals following the approach described in Strandh et al. (2012). A maximum-likelihood tree was constructed based on the chemical binding properties of the amino acids, as described by five physico-chemical descriptor variables (z-descriptors) for each amino acid, using sequences of *Meles meles*, *Meles leucurus*, *Meles anakuma* and *Martes zibelina* as out-group retrieved through NCBI blast (Figure S1). The trees were used as reference from which the functional distances between individuals were calculated using unweighted UniFrac for both genes (Lozupone & Knight, 2005). Following Bolnick et al. (2014), the genetic distance between each amino acid sequences within each individual (Faith's PD) were calculated, and further defined as motif divergence.

5.3.4 Statistical analysis for MHC and microsatellites markers between populations

For subsequent analyses, individuals were sorted according to their population origin (western and eastern) that corresponded to structure results (admixed individuals ($q < 0.9$) were excluded = 0 individuals). Non-parametric Kruskal-Wallis rank sum tests were used to investigate neutral genetic variation with F and MLH calculated from microsatellite markers analysis, between the two E-mink populations and host sex. Adaptive genetic diversity and variation between E-mink populations and individual sex was also observed using the same approach for motif richness for both MHC genes and divergence.

Permutational multivariate analyses of variance (PERMANOA) models *adonis* from the vegan package were constructed with 9,999 permutations with reported F, R2, and p- values, to determine whether there were significant differences in genetic distance matrices for neutral and adaptive markers between E-mink population, individual sex and birth location. Pearson's pairwise correlation tests between the presence of MHC motifs, present in at least three individuals, were conducted to potentially represent haplotype blocks for the two genes investigated. The significance cutoff was set to p-value < .05 for each test. Mantel tests were also conducted between each neutral, MHC-I and MHC-II genetic distance matrices with 1,000 permutations to investigate correlation between each marker.

5.3.5 Microbiota data generation and processing

After DNA extraction, the targeted gene for bacterial taxonomic affiliation using broad bacterial primers of the region V4 of the 16S rRNA gene (515F-806R, 390 bp) was amplified through PCRs. Amplification, library preparation and sequencing were carried out in a similar manner to MHC genotyping, with a depth of 100,000 reads/samples of two libraries composed of 48-52 samples.

The quality controls of the demultiplexed paired-end reads were performed through the software FastQC (Andrews, 2010). Demultiplexed sequence reads denoising and amplicon sequence variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al., 2018; v. 2019.1), using the DADA2 pipeline (Callahan et al., 2016; Callahan et al., 2017). Samples were pooled by individuals to limit bias from diet foods prior to rarefaction. Rarefaction was conducted at 27,000 reads/samples in sampling depth. ASVs—or also referred to as bacterial phylotypes—were then screened to the 97% 16S rRNA gene full-length reference sequences from the Silva v.132 database (Pruesse et al., 2007) for taxonomical association using the VSEARCH classifier implemented in QIIME2 (Bokulich et al., 2018). Sequence alignment and phylogeny building were conducted in QIIME2 for the construction of UniFrac distance matrices.

5.3.6 Statistical analysis for α-diversity of gut bacteria according to host information

All statistical analyses were conducted using the phyloseq and microbiome packages for manipulation of data. Total observed number of bacterial taxa, Chao1, Shannon's diversity index and Faith's PD in each sample were used as metrics to measure the α-diversity of gut bacteria between samples. Chao1 characterizes the overall phylotype richness within a host, Shannon's diversity index considers richness and abundance, and Faith's PD is an indicator of genetic diversity within a sample. Differences in the indices according to E-mink populations, sex and birth location of the host were analyzed using Kruskal-Wallis rank sum tests. Linear regression models were also conducted with the different measures of the microsatellites and MHC analysis (MLH, F for microsatellite data, MHC motif richness and divergence) as predictors, and microbial richness indexes as response variable. Homogeneity of variance assumptions were tested using Levene tests and normality of the residuals with Shapiro-Wilk tests and visual representations.

5.3.7 Statistical analysis for β-diversity of gut bacteria between population and differential abundance

Weighted and unweighted UniFrac distance matrix between samples were used to investigate differences in gut microbial communities between E-mink population and host sex. These metrics consider the differences in phylogenetic distance and abundance of each bacterial community between samples, pairwise, emphasizing on rare (unweighted) or abundant microbial taxa (weighted). PERMANOVA tests were conducted in similar conditions than PERMANOVA tests conducted on MHC matrixes. Additionally, models were constructed with adaptive genetic variables: motif richness and divergence from both MHC genes. To test the hypothesis that both neutral and adaptive distances are correlated with gut microbial composition, we employed Mantel tests between each genetic distance and Unifrac distance.

A differential abundance analysis was conducted on the raw ASVs count (after filtering, prior to rarefaction) that were present in more than 10% of all the samples and that had a relative abundance of more than 5% among all taxa. It corresponds to the core microbiota of the dataset, represented by 1203 phylotypes. The abundance analysis was made at the ASV level with the DESeq2 package, using a negative binomial Wald test to test significance in contrast between each E-mink population and each common MHC motifs that were present in at least two individuals. Only microbial ASV with a significance level (α) below .001 after false discovery rate (FDR) corrections were considered using the Benjamini–Hochberg method (Love et al., 2014). From the same core microbiota, I also tested for Pearson correlations between microbial genera' relative abundance per individual and continuous genetic variables

(MLH, motif richness and PD for both MHC class I and II) with FDR corrections, for genera that were encountered at least in three individuals.

5.4 Results

5.4.1 Genetic diversity of the European mink captive populations

The microsatellite markers analysis demonstrated an overall population allelic richness per locus of 2.69 with an average of 2.49 in the western population, and 2.82 in the eastern population. Heterozygosity values were lower in the western population compared to eastern population (Table 1). Bayesian assignment recovered two genetic clusters within our population, and no admixture pattern were detected. All individuals clustered according to populations, corresponding to the two different breeding facilities. The offspring with parents of each population was assigned to the western population according to clustering ($p(K_{western})=0.975$). Multilocus heterozygosity was slightly higher in eastern than western populations (Kruskal-Wallis: $\chi^2=3.4761$; p-value=0.0623), but the inbreeding coefficient (F) was not (Kruskal-Wallis: $\chi^2=0.085714$; p-value=0.7697). Overall, sex and birth location had no significant effect on neutral markers' diversity and richness. PERMANOVA on genetic distance based on the microsatellite markers detected no variation according to E-mink population, sex and birth location (Table 2).

Raw MHC amplicon sequencing data consisted of 3,084,478 raw reads with an average length of 230 base pairs for MCH-I and 130 base pairs for MHC-II. After processing, I characterized 13 MHC-I motifs (amino acid sequences) and 6 MHC-II motifs. The average number of motifs per individual was 5.3 and 3.08 (range: 2-9; 2-4) for MHC-I and MHC-II genes respectively, indicating the presence of at least five and two copies for the two regions. For the MHC-I gene, three motifs were strictly present in the eastern E-mink, and one motif in the western E-mink. Comparatively, no motif were unique to eastern E-mink for MHC-IIex2 gene, and three were strictly found in western E-mink (Figure 1). Spearman correlation tests allowed us to detect haplotype blocks for both genes, mostly attributed to the eastern population (with the motifs Mulu:MHC-I*0003, Mulu:MHC-I*0008, Mulu:MHC-I*0012, Mulu:MHC-I*0013 and Mulu:MHC-I*0015 for MHC-I and Mulu:DRB*90701, EU263553 for MHC-II) and western E-mink (Mulu:MHC-I*0007, Mulu:MHC-I*0009 and Mulu:MHC-I*0011 for MHC-I, KM371114_EU263551, EU263558_LC055119, EU263550_EU263557 and EU263554_EU263552_EU263556 for MHC-II, Figure S2 & Table S2). Most of the variation encountered in both genes was expressed in amino acid residues that influence the binding of CD4 and CD8 glycoproteins involved in antigen presentation for adaptive immunity (Figure S3).

Motif richness and divergence (Faith's PD) were significantly higher in the western E-mink compared to eastern E-mink for MHC-II gene (Kruskal-Wallis: χ^2=13.456, p-value=0.0002; χ^2=8.0614, p-value=0.0045; respectively). However, for MHC-I, divergence was higher in eastern E-mink compared to western E-mink, but not motif richness (Kruskal-Wallis: χ^2=5.0097, p-value=0.0252; χ^2=1.5456, p-value=0.2138, respectively). No changes in motif richness nor divergence were observed according to sex for the two genes. However, I did observe significant variation in MHC-II richness according to birth location (Kruskal-Wallis: χ^2=10.854, p-value=0.0125), and a Dunn test with Benjamini-Hochberg correction only detected higher motif richness for the MHC-II gene in captive-born E-mink in Spain compared to the EEP (Dunn: Z=-2.748, adjusted p-value=0.0358). PERMANOVA detected a significant influence of E-mink sex for MHC-I genetic distance, as well an influence of mink population close to the significance threshold (Figure S5), whereas E-mink population was the only variable influenced MHC-II composition variation (Table 2). Finally, Mantel tests showed a positive correlation between MHC - I and neutral markers distances (Mantel: r=0.2761, p-value=0.001).

5.4.2 A-diversity of gut bacteria according to host information

A sample of mock community containing known concentrations of genomic DNA from 20 bacterial strains was sequenced. 19 of the 20 different strains originally included in the sample were detected. The undetected strain was present at the lowest concentration. Therefore, our protocol allowed bacterial DNA detection and identification to the genus level as long as its concentration in the DNA extract was at least 2.8 pg/µl, and provided that the sequence was included in the reference database.

After reads processing, a total read count of 624,796 was obtained for gut microbial communities in captive E-mink, with an average counts per sample of 26,033 after rarefaction to limit sequencing depth artifacts. A total number of 5703 ASVs – or phylotypes – were distinguished in the samples. The gut microbiota of the E-mink was mostly composed of the *Firmicute* phylum (74%), which was dominated by the *Clostridiaceae* and *Peptostreptococcaceae* families, followed by *Proteobacteria* (14%) with *Enterobacteriaceae*, *Moraxellaceae* and *Pseudomonadaceae* families (Figure 2).

Despite an overall observation of lower microbial richness in the western compared to the eastern populations, no significant results were observed in multiple microbial richness indices (Shannon index, Chao1; Figure 3). However, I did observe slightly lower Faith's PD in western compared to eastern individuals (χ^2= 2.8834, p-value = 0.0895). Western females had significantly lower microbial phylogenetic diversity compared to males (Figure 3). Despite not reaching statistical significance (R^2=0.1626, F=0.5502, p-value=0.7633), linear regression

models with alpha diversity measures as response variables showed negative correlations with adaptive genetic richness measures, and particularly strong estimates for MHC-I richness (Figure S4).

5.4.3 B-diversity of gut bacteria between E-mink and differential abundance

Beta diversity analyses revealed that bacterial communities were significantly different in composition according to E-mink population only when considering unweighted Unifrac distances, whereas no significant differences in microbial community composition were found between host sexes nor birth locations (Models 4 and 6, Table 2). Moreover, MHC-II gene richness had a small significant influence on gut microbial composition (Model 5, Table 2). This is reflected in the results of the PCoA, which clustered individuals with differences in number of MHC-II motifs (Figure 4). Mantel tests also shown a significant positive correlation between unweighted Unifrac distance and MHC-II genetic distance (Mantel: r=0.4811, p-value=0.019), and despite not reaching statistical significance, a negative correlation with MHC-I genetic distance (Mantel: r=-0.0823, p-value=0.862) and close to zero for neutral markers' distance (Mantel: r=0.0065, p-value=0.229).

I recovered several bacterial genera and families whose relative abundances were significantly correlated with MHC-I and MHC-II richness and divergence, while some marginally correlated with multilocus heterozygosity of neutral markers (Table 3). A majority (65%) of the Pearson correlations appeared to be negative between bacterial genera and genetic indexes. Differential abundance analysis in microbial families according to presence of MHC motifs for both genes between E-mink populations detected a significant increase in abundance for 13 families in the eastern population, for 8 in western E-mink (Figure 5).

From the differential abundance analysis, 22 phylotypes were found to be significantly different in abundance according to MHC motifs presence. MHC-I motifs explained the variation of 3 phylotypes, and only impacted the *Clostridiaceae* family (Clostridium sensu stricto 1). MHC-II motifs presence was attributed to the altered the abundance of 14 microbial phylotypes and those were mostly more abundant in the eastern E-mink as 13 phylotypes were more abundant in eastern E-mink against one in the western E-mink. MHC-II motifs impacted over twelve genera compared to one for MHC-I motifs. It is also worth noting that both MHC-I and MHC-II motifs presence were observed to alter taxa abundance for 5 phylotypes, mostly from the Proteobacteria phylum (Table 4). The MHC-II motifs KM371114_EU263551, EU263554_EU263552_EU263556, and Mulu:DRB*90701 respectively took part in the variation in abundance for 17, 15 and 12 phylotypes respectively. MHC-I motif Mulu:MHC-I*00008 was significantly involved in the variation for 4 phylotypes, whereas the

other motifs had relatively low impact, as they took part in abundance variation of 1-2 families.

5.5 Discussion

5.5.1 Genetic variation in the two E-mink captive breeding programs

Microsatellite markers for both populations exhibited low allelic richness and heterozygosity indices, with the western population having the lowest values, in line with previously published results (Michaux et al., 2005; Cabria et al., 2007; Cabria et al. 2015). However, the eastern E-mink sampled in this study might not reflect the full genetic variation within the entire population, as collected E-mink originated from a subset of the EEP since its start 25 years ago (Becker et al., 2009). Conversely, western E-mink sampled came from wild-born and captive-born individuals from a recent breeding program. Our Bayesian clustering analysis suggests the existence of at least two main genetic units of E-mink defined by their origin with the captive programs, validating our use of the two E-mink groups for studying their genetic and gut microbial variation.

Nonetheless, the two MHC genes investigated revealed differential variation between the two E-mink populations, the MHC-I gene being more divergent in eastern E-mink and the MHC-II gene exhibiting more richness and divergence in western E-mink. Interestingly, the adaptive genetic diversity followed the neutral markers trend only for one gene and not the other, making the assessment of genetic diversity in captive breeding complex. The maintenance of genetic variation in neutral markers through non-selective evolutionary forces (genetic drift, inbreeding) depend on the number of founders in a population, as well as the breeding system of the species. However, balancing selection is believed to counteract those non-selective evolutionary forces in functional genes (Hedrick, 1999), resulting in an excess of heterozygotes in small, isolated populations for MHC-II loci. This pattern has been observed in several isolated populations (Aguilar et al., 2004; Jarvi et al., 2004; Schad et al., 2004), but all species investigated were free ranging, implying less restrictions in the mating system compared to CBPs and therefore stronger sexual selection.

In line with previous evidence of the role of sexual selection for MHC pattern distribution in vertebrates (Edwards & Hedrick, 1998), I observed that sex had an influence on MHC-I gene composition. It has been shown that MHC class I genes may be involved in pheromone recognition, and that mate preferences can be reflected in dissimilarity of MHC patterns (Penn, 2002). In the case of the E-mink, captive-bred males are less successful breeders compared to wild-born males (Kiik et al., 2013). Therefore, mate pairing based only on pedigree might not provide enough information and might be hindered by MHC-I

similarities between potential mates. Variation at neutral markers may thus not accurately reflect variation at potentially relevant genes, particularly those under selection like the MHC (Ujvari & Belov, 2011), and a global genetic assessment should be taken in consideration in conservation genetics for management decisions (Mardsen et al., 2013).

5.5.2 The ecosystem on a leash model in mammalian gut microbiota

The hypothetical framework presented by the ecosystem on a leash model (Foster et al., 2017) suggests that more host control in distantly related microbes, illustrated by the Faith's PD index and Unifrac distances, should be found in individuals with high MHC diversity. This pattern was observed, although weakly, in alpha diversity analysis for the western population, highlighting the importance of the MHC class II gene above MHC class I and microsatellite markers. It is also worth noting that this result only involves distantly related microbes, emphasizing the fact that a phylogenetically diverse microbiota could lead to the dominance of the fastest growing microbes instead of the microbes that are most beneficial to the host, lending support to the ecosystem on a leash model.

Similar more robust trends were found in beta-diversity analysis, where gut microbial composition was different according to the number of MHC-II motifs a E-mink possessed and the more distant two E-mink are in MHC-II haplotype, the more different in rare gut microbiota composition as well. Mostly negative correlations were observed between microbial abundance and MHC genes richness and divergence, likewise suggesting more host control in individuals with high adaptive genetic variation. This also supports an advantage in balancing selection despite strong genetic drift. The differential abundance analysis also revealed stronger host control in the western population, mostly explained by the presence of specific MHC-II motifs. Moreover, the MHC-II gene was more likely to impact a wide range of microbial taxa. These results are in line with previous studies conducted on fish, mice and birds (Bolnick et al., 2014; Khan et al., 2019; Leclaire et al., 2018). However, I did observe that one motif of MHC-II present in the eastern population also impacted the abundance of several bacterial families, indicating that the captive eastern population still possess interesting motifs for host control.

Because the MHC-I gene targets intracellular non-self-molecules recognition, it would impact a smaller number of bacteria compared to MHC-II (Ost & Round, 2018). Other taxonomic groups such as viruses and protists would need to be targeted for further investigation, and eastern population might be more equipped to recognize them, given the increased diversity for this MHC-I gene (Kubinak et al., 2012). This is of particular importance knowing the circulation of several viruses in free-ranging western E-mink (Fournier-Chambrillon et al., 2004; Philippa et al., 2008; Mañas et al., 2016). The canine distemper virus,

which results in a high mortality rate in E-mink, is currently re-emerging in many wild carnivore populations in Europe (Origgi et al., 2012) and has had a major impact on population of E-mink in Navarra, Spain (Fournier-Chambrillon et al., 2022).

Overall, our prediction that less host control will be observed in mink with lower genetic diversity is supported by both alpha and beta diversity for the E-mink. However, both populations have low genetic diversity, and the MHC class II DRB gene seemed to have a stronger influence in gut microbes than other markers. To further validate our results, replicating the study to see if those differences are observable when individuals from the two populations are kept in the same facility to control for the influence of the external environment should be conducted. Given that I only had access to samples from a small fraction of the captive eastern population, our results might also not be representative of the entire captive breeding stock. Despite the gut microbiota variation being a complex puzzle, our study gives more importance to host immunogenetics in the context of species conservation.

5.5.3 Adaptation to captivity and management practices

For MHC genes, rare allele and heterozygous advantage are two types of balancing selection that have been suggested to be important in maintaining high levels of adaptive genetic diversity (Sommer, 2005). Assuming that rare and divergent MHC genotypes are more likely to induce host control on gut microbes, giving a fitness advantage to the host, the co-evolutionary arm race with gut microbes will foster adaptation from microorganisms to not be targeted by common MHC alleles (Kubinak et al., 2012). However, microbe-driven selection could vary over time and space and between E-mink populations. This mechanism could be of influence in the western E-mink population, given that the breeding program started in 2013 and individuals from the wild are still being captured to increase founder size in the program from the natural habitat. Moreover, low MHC class II gene diversity in the eastern population might indicate that non-evolutionary forces overshadow balancing selection for this locus, which could be mainly explained by extensive constrains in the mating system for a long period of time.

Inadvertent genetic adaptation to captivity for endangered species has been documented over recent years (reviewed in Frankham, 2008). This has been related to a fitness reduction when animals are released in the wild environment, increasing with the numbers of captive-bred generations, including changes in reproductive success, morphology and behavior (Williams & Hoffman, 2009; Willoughby & Christie, 2019). Becker et al. (2009) previously investigated the MHC-II DRB gene in the captive eastern E-mink population, and detected nine alleles, representing 6 motifs. However, ten years later, I observed 3 motifs in the eastern group. The

EEP in Estonia started in 1992 and has not been supplemented by wild individuals for at least 25 generations (T. Maran, pers. comm., 2021). Moreover, given the moderate success of the breeding program due to captive-born male behavior (Kiik et al., 2013), this suggests that high number of generations in captivity led to loss of genetic diversity and deleterious genetic fixation took place for this population (Woodworth et al., 2002; Frankham, 2008; Witzenberger & Hochkirch, 2011; Parmar et al., 2017). Even though 90% of the initial gene diversity has been maintained through studbook calculations (T. Maran, pers. comm., 2021), it is likely that studbook measurements might not reflect this trend for all E-mink genes. However, different management strategies have been proposed to mitigate fitness reduction for future reintroduction (reviewed in Williams & Hoffman, 2009) that could be implemented for the E-mink.

One strategy is to translocate animals between breeding centers for reproduction to prevent loss of genetic diversity. Similar to the western captive population of E-mink, these translocations could be composed of wild-born individuals, free of captive selection pressure (Schulte-Hostedde & Mastromonaco, 2015). Occasional translocations from western to eastern captive populations could also be conducted and would potentially mitigate the modest reproductive success within the program. It is worth noting that wild-born animals have been out of reach from the EEP breeding stock so far. However, conducting preliminary MHC variation assessment on reintroduced animals from the eastern stock present in Hiiumaa island, as they no longer face captivity for a number of generations, could be used to identify potential assets to the current breeding stock.

Captivity has been shown to alter gut microbial communities (McKenzie et al., 2017). Combined with this traditional conservation efforts, microbial rescue could also help improve success of managing at-risk populations. For example, the most common cause of mortality in captive cheetahs (*Acinonyx jubatus*) is bacterial infection, possibly because of an increase in pathogenic taxa compared to wild conspecifics (Wasimuddin et al., 2017). Microbial rescue, using probiotics, can stabilize the composition of the gut microbiota of dolphin in captivity (*Lagenorhynchus obliquidens*, Cardona et al., 2018). Implementing wild-like diet-based enrichment could also mitigate captivity effects on gut microbial communities in the same way as captive selection (Mueller et al., 2019; Trevelline et al., 2019; van Leeuwen et al., 2020), but further research is needed in the E-mink case.

These types of strategies could increase adaptive genetic diversity related to immunomodulation and therefore a fitness advantage to the mink once reintroduced. Coupled with a more in-depth investigation on the gut microbiota of the E-mink according to diet and environment manipulation, these technics can have synergetic effects and foster the success of the CBPs (Gould et al., 2018; West et al., 2019). This first look into the

connection between management strategies, genetic diversity and gut bacteria within the CBPs of the E-mink allowed preliminary assessment of the current situation. It also offers many axes of further research and potential strategies with the on-going challenges that many *ex situ* conservation programs face to mitigate species extinction.

5.6 References

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5.7 Tables and figures

	Eastern	Western	Distance between populations	
N	14	10	Gst	0.0615
A	50	43	Djost	0.0284
Ar	2.82	2.49	Fst	0.1165
Ho	0.54	0.44		
He	0.48	0.43		
Fis	-0.1241	-0.0403		
Fis_Low	-0.252	-0.2265		
Fis_High	-0.0177	0.1047		

Table 1. Measures of neutral genetic diversity through microsatellite marker analysis by E-mink captive population (Eastern = EEP breeding center in Zoodysée, France; Western = FIEB breeding center in Spain). N: number of individuals, A: total number of alleles, Ar: mean allelic richness per locus, Ho: observed heterozygosity, He: expected heterozygosity, Fis: mean inbreeding coefficient. Overall mean value for each population across the 16 markers.

Model	Distance matrix	Variables	F statistic	R squared	P-value
1	Microsatellite	Mink population	1.45837	0.06286	0.2318
		Birth location	1.42653	0.06149	0.2390
		Sex	0.77162	0.09978	0.7236
		Residuals		0.77587	
2	MHC-I	Mink population	2.8442	0.09820	0.0797
		Birth location	0.9910	0.10264	0.4476
		Sex	**5.1459**	**0.17767**	**0.0200 (0.387)**
		Residuals		0.62148	
3	MHC-II	**Mink population**	**5.4984**	**0.21090**	**0.0284(0.418)**
		Birth location	0.7741	0.08908	0.5412
		Sex	0.2503	0.00960	0.7140
		Residuals		0.69042	
4	Microbiota (UWU)	**Mink population**	**1.44739**	**0.06203**	**0.0003 (0.229)**
		Birth location	0.96248	0.12375	0.7692
		Sex	0.99747	0.04275	0.4722
		Residuals		0.77146	
5	Microbiota (UWU)	MLH	0.97674	0.04194	0.5673
		MHC-II divergence	1.06218	0.04561	0.2064
		MHC-II richness	**1.21292**	**0.05195**	**0.0219 (0.821)**
		MHC-I divergence	0.99014	0.04252	0.5019
		MHC-I richness	1.10666	0.04752	0.1151
		Residuals		0.77292	
6	Microbiota (WU)	Mink population	1.26796	0.06009	0.2749
		Birth location	0.40095	0.05701	0.8163
		Sex	0.62848	0.02979	0.6250
		Residuals		0.85311	
7	Microbiota (WU)	MLH	1.24147	0.05512	0.2745
		MHC-II divergence	0.27059	0.01201	0.7573
		MHC-II richness	1.37532	0.06106	0.2411
		MHC-I divergence	0.57734	0.02563	0.6026
		MHC-I richness	1.05875	0.04701	0.3326
		Residuals		0.79917	

Table 2. Model results of PERMANOVAs for 9,999 permutations for each distance matrix according to variables of interest. Values in bracket are p-values from Marti Anderson's PERMDISP2 procedure for the analysis of multivariate homogeneity of group dispersions (variances). UWU: Unweighted Unifrac, WU: Weighted Unifrac distances.

Genus (family)	Pearson's rho	FDR p-value
Multilocus heterozygosity (neutral markers)		
Clostridium sensus stricto 13 (Clostridiaceae)	0.365	*0.065*
Epulopiscium (Lachnospiraceae)	0.410	0.034
Gemmela (Gemellaceae)	-0.352	*0.077*
Providencia (Morganellaceae)	0.367	*0.063*
MHCI ex2 richness		
Helicobacter (Helicobacteraceae)	-0.400	0.040
Staphylococcus (Staphylococcaceae)	-0.386	0.049
MHCI ex2 divergence (PD)		
Aeromonas (Aeromonadaceae)	0.382	*0.065*
Helicobacter (Helicobacteraceae)	-0.436	0.033
Paeniclostridium (Peptostreptococcaceae)	-0.384	*0.064*
Pseudomonas (Pseudomonadaceae)	0.473	0.019
Staphylococcus (Staphylococcaceae)	-0.467	0.021
MHCII ex2 richness		
Actinomyces (Actinomycetaceae)	-0.438	0.022
Luteolibacter (Rubritaleaceae)	0.366	*0.064*
Sporosarcina (Planococcaceae)	0.398	0.048
Streptococcus (Streptococcaceae)	-0.395	0.043
MHCII ex2 divergence (PD)		
Helicobacter (Helicobacteraceae)	-0.456	0.025
Lysinibacillus (Planococcaceae)	-0.632	0.0035
Staphylococcus (Staphylococcaceae)	-0.540	0.046
Streptococcus (Streptococcaceae)	-0.711	0.0001
Terrisporobacter (Peptostreptococcaceae)	-0.516	*0.063*

Table 3. Genera that were significantly correlated with or differed across variables associated with gut microbial communities. Italicized P-values were marginally significant after False Discovery Rate (FDR) correction of p-values.

				Increased abundance in:	Eastern mink			Western mink		
Bacteria Taxonomy				Explained by presence of:	MHC-I motifs	MHC-II motifs	Both	MHC-I motifs	MHC-II motifs	Both
Phylum	Class	Order	Family	Genus						
Campilobacterota										
	Campylobacteria	Campylobacterales	Helicobacteraceae	Helicobacter		1				
Firmicutes										
	Bacilli	Bacillales	Planococcaceae	Sporosarcina					1	
		Mycoplasmatales	Mycoplasmataceae	Mycoplasma						1
	Clostridia	Clostridiales	Clostridiaceae	Clostridium_sensu_stricto_1	2			1		
				Clostridium_sensu_stricto_13		1				
		Lachnospirales	Lachnospiraceae	Epulopiscium		1				
		Peptostreptococcales-Tissierellales	Peptostreptococcaceae	Romboutsia		1				
Proteobacteria										
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera		1				
		Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas			1			
			Shewanellaceae	Shewanella		2				
		Enterobacterales	Enterobacteriaceae	Escherichia-Shigella		1				
		Oceanospirillales	Unassigned	Unassigned		1				
		Pseudomonadales	Moraxellaceae	Acinetobacter		2				
			Pseudomonadaceae	Pseudomonas		2	1			
		Vibrionales	Vibrionaceae	Vibrio			1			
		Xanthomonadales	Xanthomonadaceae	Luteimonas						1
				Total by gene and population	2	13	3	1	1	2

Table 4. Summary of significantly enriched phylotypes among the two mink group according to presence of each MHC motif in each class I and class II genes from the DESEq2 analysis. If multiple motifs explained the variation of one ASV, there were accounted for once if it varied only according to motifs from the same gene.

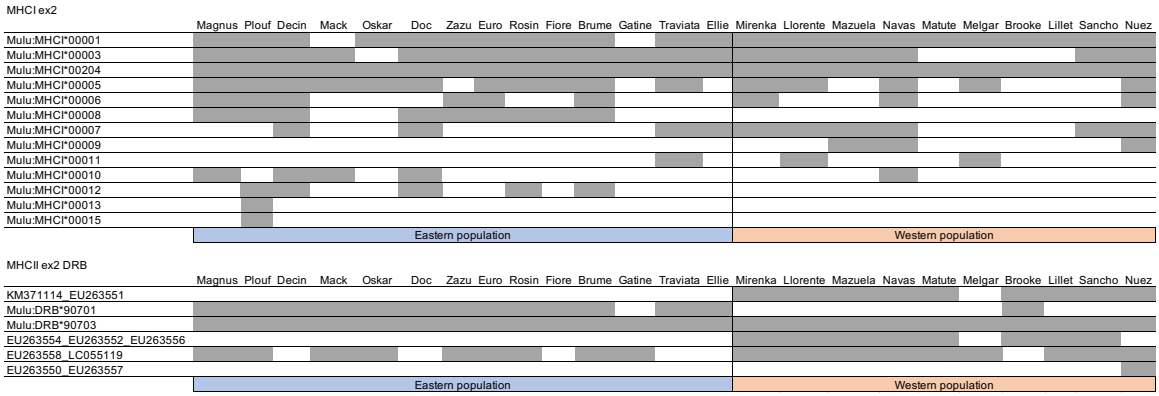

Figure 1. Distribution of MHC motifs in both mink population. Grey shading shows the presence of each motif for each individual. Newly discovered motifs were named following the nomenclature, and already identified motifs were named according to the accession number found through NCBI blast.

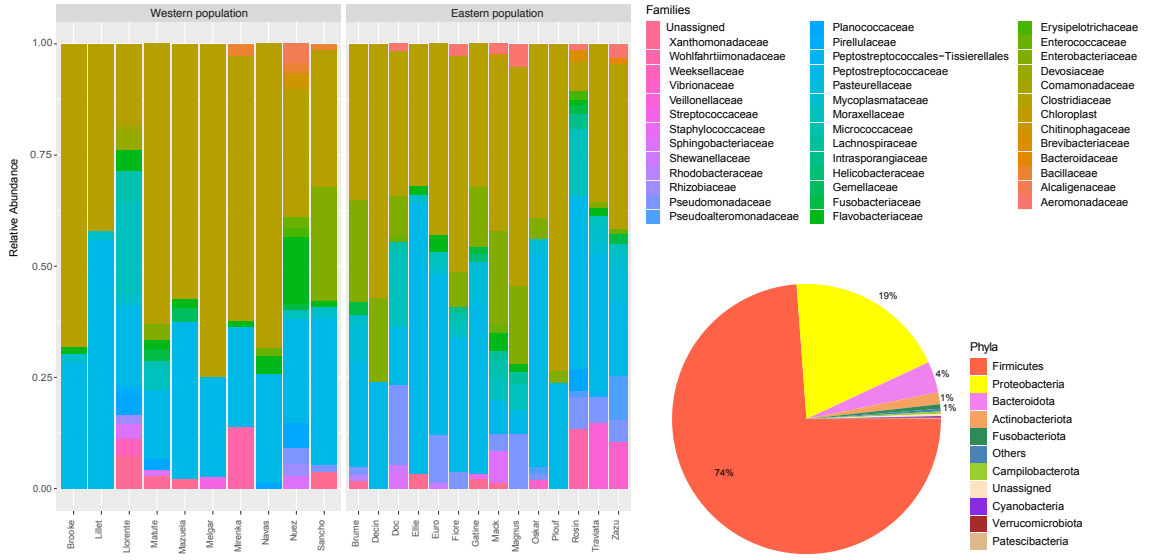

Figure 2. The relative abundance of common bacterial families across 24 E-mink individuals from the two breeding populations. The pie chart represents the mean proportion of relative abundance for each microbial phylum for all individuals.

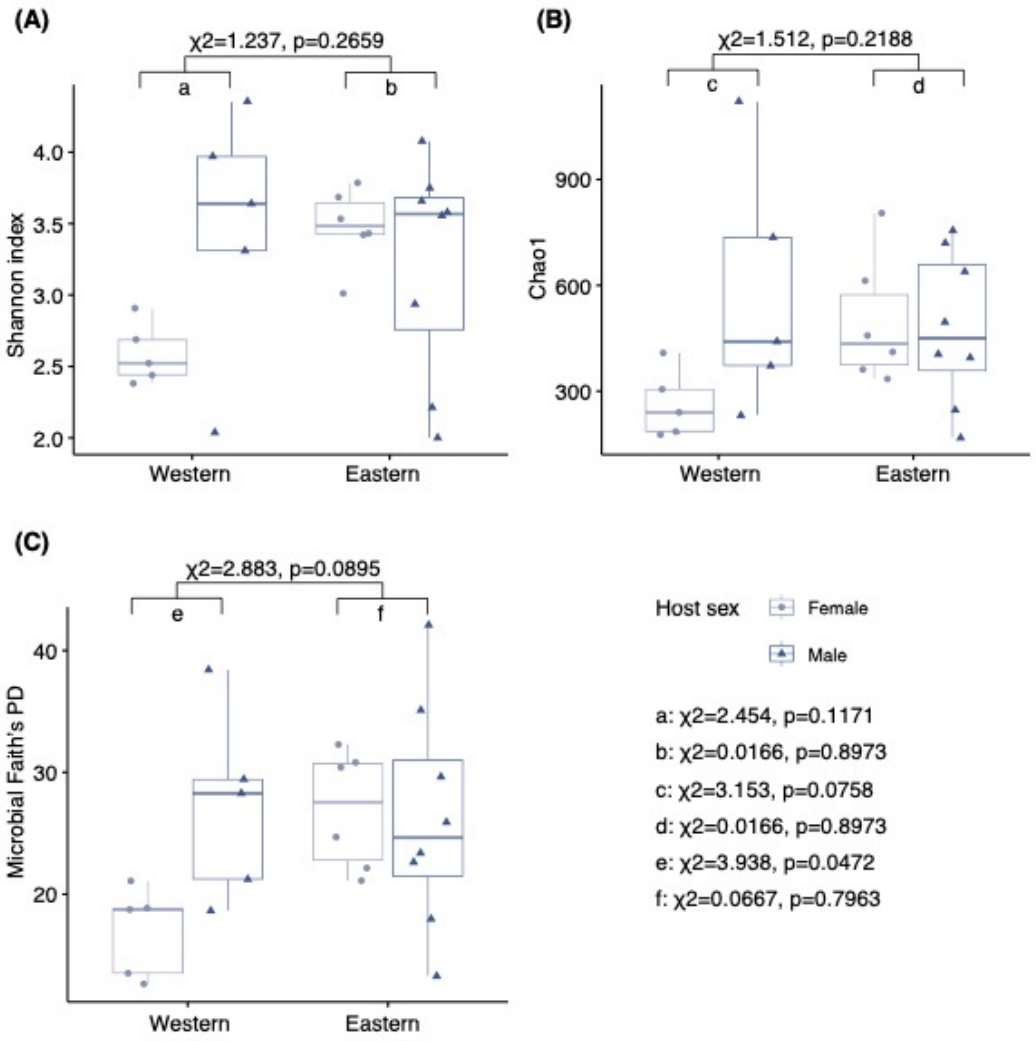

Figure 3. Alpha diversity indexes for microbial taxa richness are shown as boxplots (with median, interquartile range (IQR) and whiskers extending to the last data points). Individual values appear as light blue dots for the female mink and dark blue triangle for the males, for each mink populations. Shannon index (A) expresses changes in taxa richness and abundance, Chao1 (B) variation in strict richness, and Faith's PD (C) for phylogenetic diversity. Results from respective Kruskal-Wallis tests are documented.

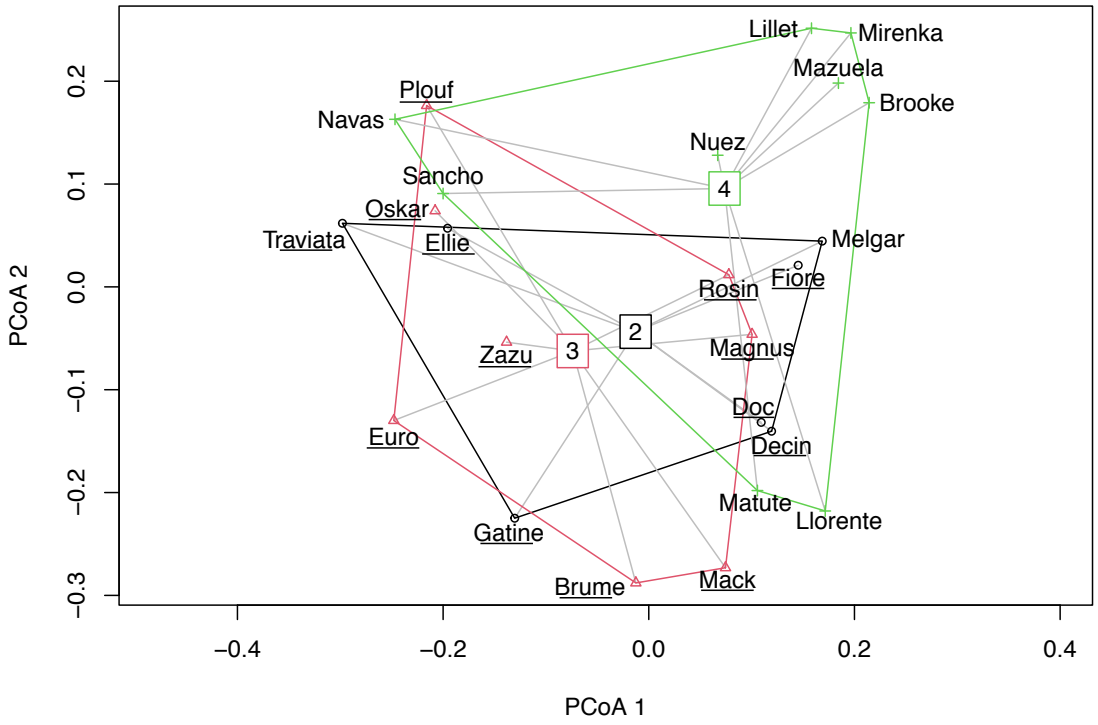

Figure 4. Differences in E-mink microbial community composition visualized by principal coordinates analysis of unweighted UniFrac distances based on rarefied OTU counts. Individuals are labelled according to their name their population or origin (animal names from the eastern population are underlined, not underlined for western). Clusters correspond to number of MHC-II motifs for each individual where the value is indicated at the centroid.

5.8 Supplementary materials

Animal name	Sex	Birth date	Captive facility	Place of birth	Population
Brooke	F	18-05-25	FIEB	Estonia captivity	Western X Eastern
Lillet	F	16-05-27	FIEB	Spain captivity	Western
Llorente	M	17-06-01	FIEB	Spain wild	Western
Matute	M	17-05-16	FIEB	Spain captivity	Western
Mazuela	F	17-05-16	FIEB	Spain captivity	Western
Melgar	M	17-05-16	FIEB	Spain captivity	Western
Mirenka	F	17-05-26	FIEB	Spain captivity	Western
Navas	F	18-05-31	FIEB	Spain captivity	Western
Nuez	M	18-06-06	FIEB	Spain captivity	Western
Sancho	M	18-06-01	FIEB	Spain wild	Western
Brume	F	19-07-10	Zoodyssée	France captivity	Eastern
Decin	M	18-05-24	Zoodyssée	Estonia captivity	Eastern
Doc	M	16-05-14	Zoodyssée	Estonia captivity	Eastern
Ellie	F	15-06-05	Zoodyssée	Estonia captivity	Eastern
Euro	M	19-07-10	Zoodyssée	France captivity	Eastern
Fiore	F	18-05-15	Zoodyssée	Estonia captivity	Eastern
Gatine	F	19-07-10	Zoodyssée	France captivity	Eastern
Mack	M	16-05-17	Zoodyssée	Estonia captivity	Eastern
Magnus	M	14-05-02	Zoodyssée	Estonia captivity	Eastern
Oskar	M	16-05-14	Zoodyssée	Estonia captivity	Eastern
Plouf	M	19-07-10	Zoodyssée	France captivity	Eastern
Rosin	F	13-05-23	Zoodyssée	Estonia captivity	Eastern
Traviata	F	19-05-22	Zoodyssée	Estonia captivity	Eastern
Zazu	M	15-05-11	Zoodyssée	Estonia captivity	Eastern

Table S1. List of animals sampled

		Prevalence	
	Motif (aa sequence)	Western	Eastern
MHCI	Mulu:MHCI*00001	100	85.7
	Mulu:MHCI*00003	60	92.8
	Mulu:MHCI*00204	60	78.5
	Mulu:MHCI*00005	50	78.5
	Mulu:MHCI*00006	30	42.8
	Mulu:MHCI*00008	0	64.2
	Mulu:MHCI*00007	60	28.5
	Mulu:MHCI*00009	30	0
	Mulu:MHCI*00011	20	7.1
	Mulu:MHCI*00010	10	28.5
	Mulu:MHCI*00012	0	35.7
	Mulu:MHCI*00013	0	7.1
	Mulu:MHCI*00015	0	7.1
MHCII	KM371114_EU263551	90	0
	Mulu:DRB*90701	10	92.8
	Mulu:DRB*90703	100	100
	EU263554_EU263552_EU263556	80	0
	EU263558_LC055119	90	64.2
	EU263550_EU263557	10	0

Table S2. Prevalence (%) of MHC motifs found in the mink population (n=10; n=14, respectively). Dark grey shading highlights motifs strictly found in one population, and light grey shading shows higher prevalence of each motif in one group.

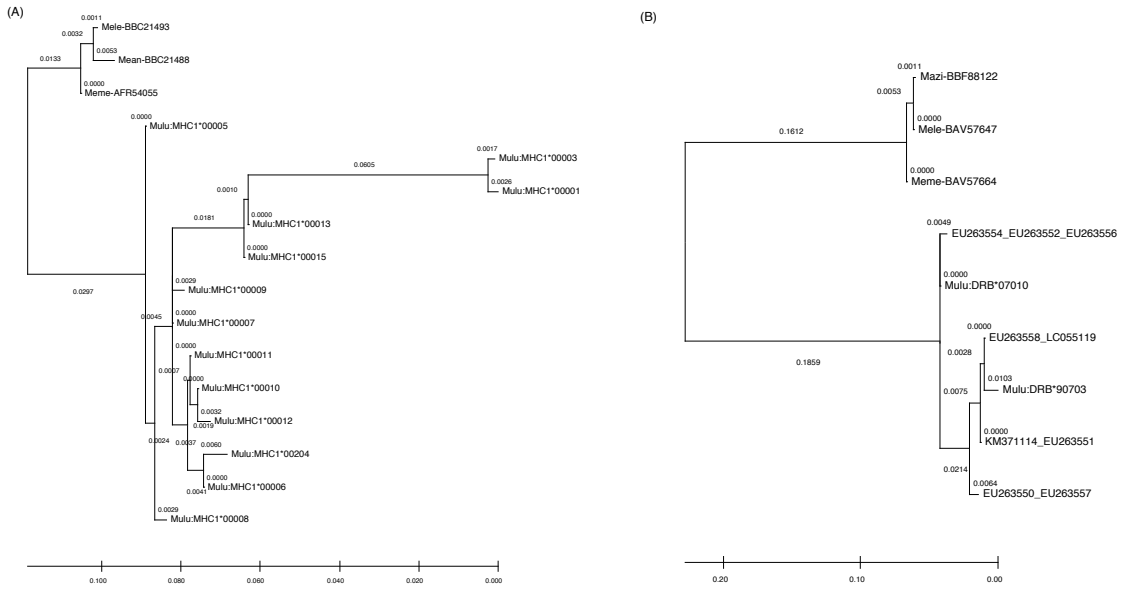

Figure S1. Maximum-likelihood trees constructed based on the chemical binding properties of the amino acids in the peptide binding regions, as described by five physico-chemical descriptor variables (z-descriptors) for each amino acid, for (A) MHC-I exon 2 motifs and (B) MHC-II DRB motifs.

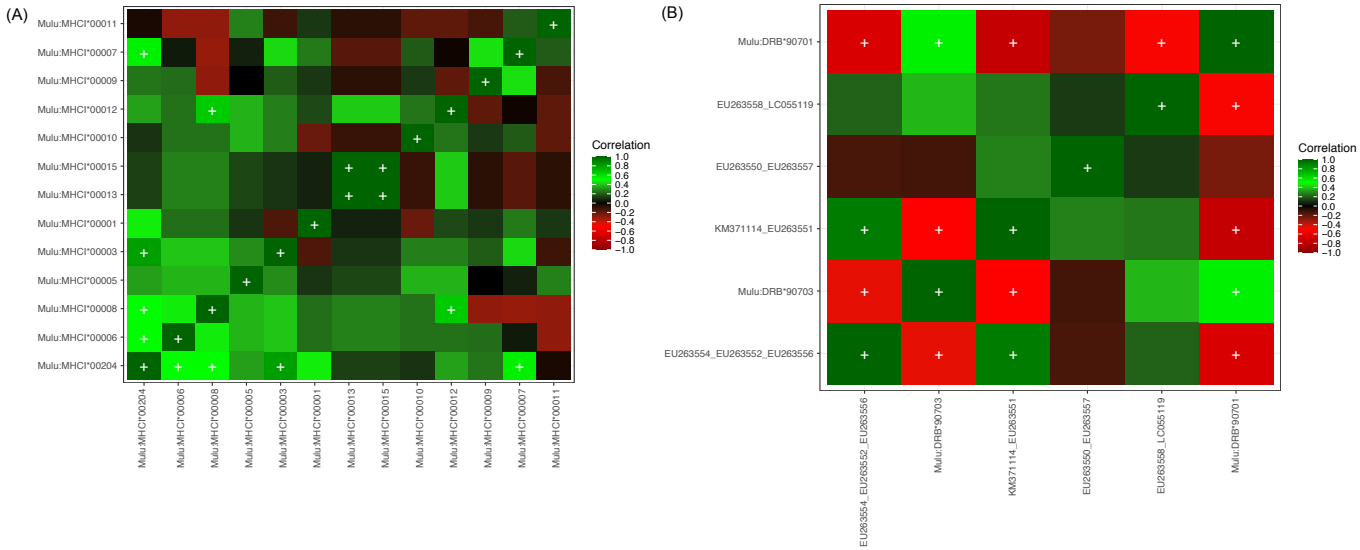

Figure S2. Spearman pairwise correlations between the presence/absence of common motifs in (A) MHC-I exon 2 and (B) MHC-II DRB exon 2. Positive correlations are colored in green, and red for negative correlations, white plus signs denote significant correlations (P-value < 0.05). Correlated motifs are likely to represent haplotype blocks among populations.

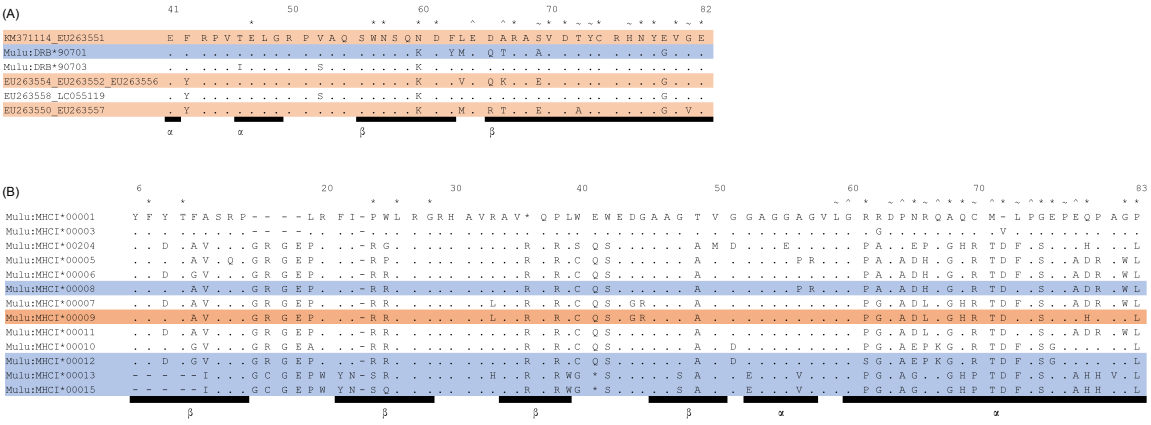

Figure S3. Alignment of partial (A) MHC class I exon 2 and (B) MHC class II DRB exon 2 amino acid sequences of *M. lutreola*. Orange shading represent motifs only present in western mink and blue shading to the eastern population. Asterisks (*) represent amino acid residues pointing toward the T-cell receptors, carets (^) highlight antigen-binding site residues pointing up on an alpha-helix, postulated to interact with peptides and/or T-cell receptors. Tildes (~) indicate residues on an alpha-helix that is pointing away from the antigen-biding sites, postulated to interact with T-cell receptors. Residues that form the beta-sheet or alpha-helix, and residues that influence the binding of the CD8/CD4 glycoprotein are marked under the alignment for MHC-I and MHC-II respectively (Sin et al., 2012; Bjorkman & Parham, 1990; Reche & Reinherz 2003).

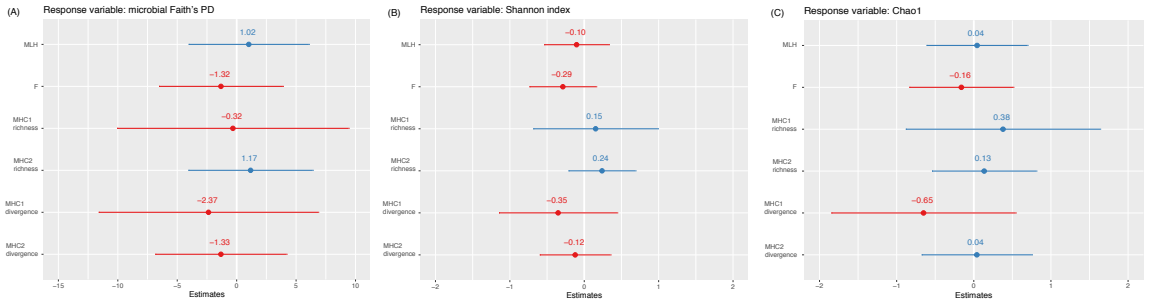

Figure S4. Distribution of estimates for three microbial richness indexes (A: Faith's PD, B: Chao1, C: Shannon index) for linear regression models with neutral and adaptive genetic diversity measures. Dots represent the mean for each estimate and whiskers extending to the last data points, in red are negative correlations and blue for positive. MLH: Microsatellite

markers' multilocus heterozygosity, F: Microsatellites inbreeding coefficient, MHC-I and MHC class II richness and divergence. A: F-statistic: 0.5502, R-squared: 0.1626, p-value: 0.7633, B: F-statistic: 0.5795, R-squared: 0.1698, p-value: 0.7417, C: F-statistic=0.5082, R-squared: 0.1521, p-value=0.7939.

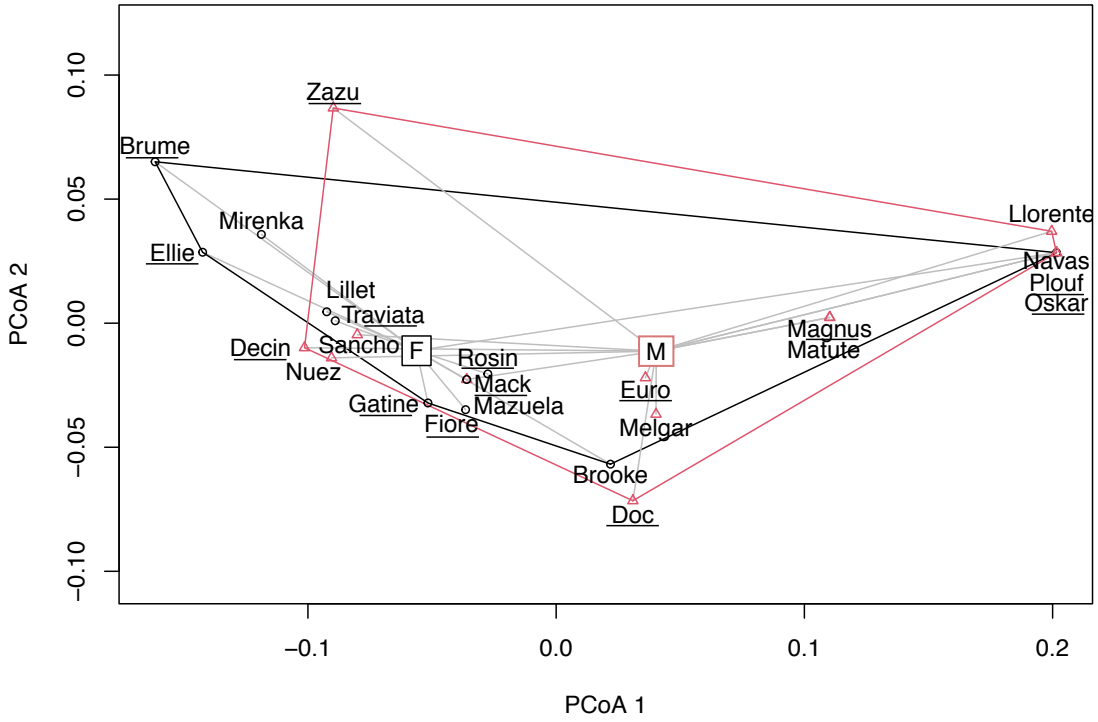

Figure S5. Differences in E-mink MHC-I motif composition visualized by principal coordinates analysis of unweighted UniFrac distances. Individuals are labelled according to their name their population or origin (animal names from eastern population are underlined, not underlined for western). Clusters correspond to the sex of each individual where the value is indicated at the centroid.

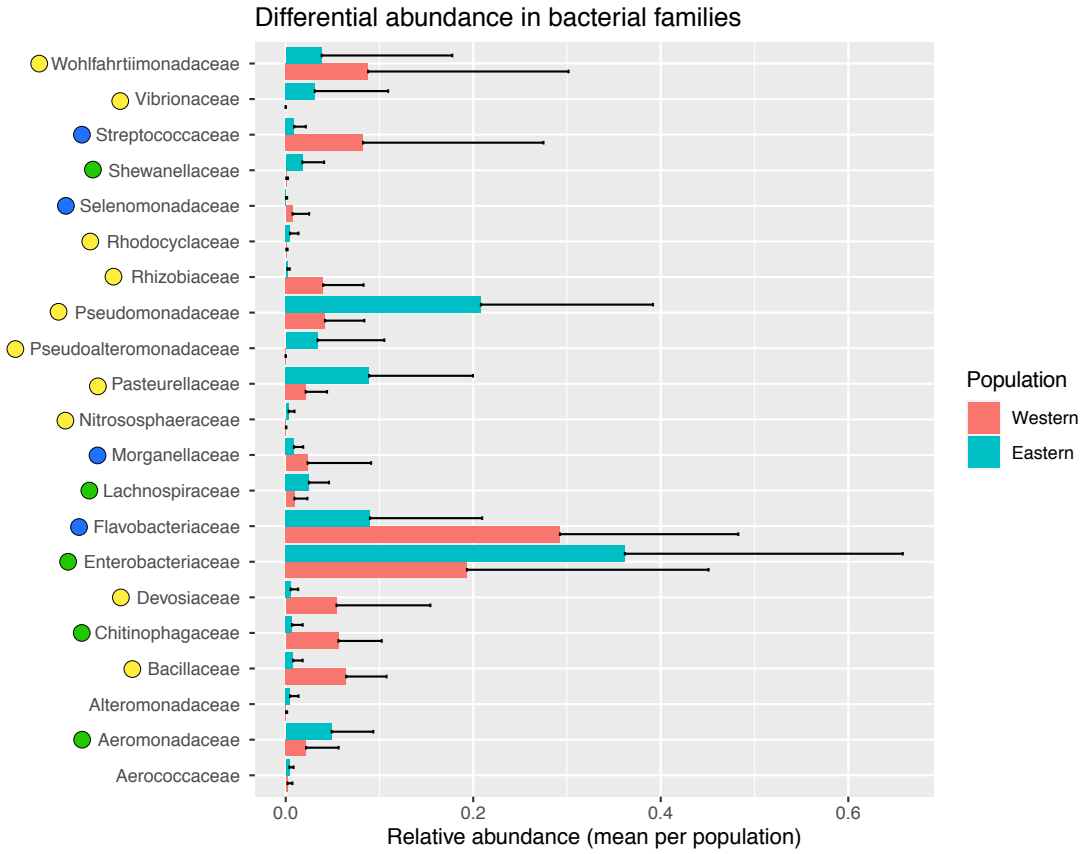

Figure 5. Mean relative abundance of each family that experienced significant differential abundance between the two mink populations (orange: Western; blue: Eastern, mean relative abundance per population + standard error). Colored circles next to the family name correspond to which MHC motifs absence/presence the variation in abundance was significant for (yellow: MHCII, blue: MHCI, green: both MHCI and MHCII).

Chapter 6. Effects of captivity, diet and relocation on the gut bacterial communities of white-footed mice

6.1 Summary

Microbes can have important impacts on their host's survival. Captive breeding programs for endangered species include periods of captivity that can ultimately have an impact on reintroduction success. No study to date has investigated the impacts of captive diet on the gut microbiota during the relocation process of generalist species. This study simulated a captive breeding program with white-footed mice (*Peromyscus leucopus*) to describe the variability in gut microbial community structure and composition during captivity and

relocation in their natural habitat, and compared it to wild individuals. Mice born in captivity were fed two different diets, a control with dry standardized pellets, and a treatment with non-processed components that reflect a version of their wild diet that could be provided in captivity. The mice from the two groups were then relocated to their natural habitat. Relocated mice that had the treatment diet had more phylotypes in common with the wild-host microbiota than mice under the control diet or mice kept in captivity. These results have broad implications for our understanding of microbial community dynamics and the effects of captivity on reintroduced animals, including the potential impact on the survival of endangered species. This study demonstrates that *ex situ* conservation actions should consider a more holistic perspective of an animal's biology including its microbes.

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Effects of captivity, diet, and relocation on the gut bacterial communities of white-footed mice

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6.2 Introduction

The reintroduction and relocation of individuals in the context of species conservation faces many challenges (Fischer & Lindenmayer, 2000; Seddon et al., 2007; Game et al., 2014), including the fact that individuals released from captive breeding programs often struggle to thrive in their natural habitats (Gilbert et al., 2017; Willoughby & Christie, 2019). These difficulties may be caused by adaptations acquired from generations of captivity (Snyder et al., 1996, Schulte-Hostedde & Mastromonaco, 2015), and disease (Viggers et al., 1993; Kołodziej-Sobocińska et al., 2018) and/or the inability to transition to a native diet (Kleiman, 1989; Jules et al., 2008).

It has been increasingly recognized that host-associated microbes should be considered in wildlife management practices, particularly in the context of conservation (Redford et al., 2012; Amato, 2013; Bahrndorff et al., 2016; Stumpf et al., 2016; Trevelline et al., 2019; West et

al., 2019). Animals provide numerous ecological niches for microorganisms, such as bacteria, archaea, protozoa, fungi and viruses. These communities of microbes in a host are collectively known as the microbiota. The gut microbiota can play a role in host development, digestion, immunity, and behavior (Suzuki, 2017; McKenney et al., 2018) and can therefore influence the survival of relocated animals. Host-associated microbiota are highly dynamic communities and disrupting their equilibrium can lead to negative direct or indirect effects on their host (Hooks & Malley, 2017; reviewed in West et al., 2019) such as impaired immune function and metabolic disorders (Clayton et al., 2016; Krynak et al., 2017; Rosshart et al., 2017; Wasimuddin et al., 2017).

The external environment of a host (Schmidt et al., 2019), its diet, and genetics (Spor et al., 2011; Campbell et al., 2012) are all known to modify the gut microbiota. Housing facilities such as zoos where captive breeding programs are held, provide intense veterinary care, sanitized enclosures, a standardized diet, and reduced sexual selection. Captivity has been shown to alter the microbiota of animals compared to wild counterparts (Clayton et al., 2016; Borbón-García et al., 2017; McKenzie et al., 2017; Wasimuddin et al., 2017). The majority of the studies show similar trends: a decrease in bacterial phylotype richness (or α-diversity) among captive individuals compared to their wild conspecifics, as well as differences in community composition (or β-diversity) between the groups. However, some host species show an opposite pattern (McKenzie et al., 2017; Greene et al., 2019; Frankel et al., 2019), postulating that the gut microbiota of group taxa respond differently to captivity, mainly through their feeding strategy and gut physiology. Differences observed in gut microbial communities have largely been attributed to altered diets in captivity that can also lead to the extinction of microbial niches and functions in the host's gut over multiple generations in captivity (Sonnenburg et al., 2016). Standardized diets are generally composed of simple fibres in low quantity compared to carbohydrates. A loss of microbial taxa taking part in the digestion of fibres in captivity has been linked to disease as a short-term disadvantage to hosts (Amato et al., 2016; Krynak et al., 2017; Rosshart et al., 2017) and could also, in the long run, be a disadvantage when hosts are relocated to their natural habitat. In general, there is extensive gut microbiota variation when an animal fed on a more animal-based or plant-based diet for humans and mice, compared to a balanced diet from various food sources (Heiman & Greenway, 2016). The consumption of a diverse diet avoids the loss of crucial microbial function linked to a specific food item in the case of omnivorous host. This has been demonstrated through the evolution of human lifestyles. Human microbial communities have been shaped through changes from hunter-gatherer and nomad societies to farming, sedentary and urban lifestyles. Especially in Western diets, the lack of fibrous food items and the increased consumption of processed foods have resulted in a reduction of gut bacteria diversity that has been implicated in many diseases linked to impaired immune responses and metabolic disorders (Kolodziejczyk et al., 2019). It is therefore essential to study the effects of

changes in diet during captivity on the gut microbiota among different host taxa with variable ecological niches, being dietary generalists or specialists, omnivorous or herbivorous for example. Previous work has suggested that a change to a more fibrous and less processed diet in captivity changes the gut microbiota compared to a standard diet, but it does not make the gut microbiota of captive animals more similar to their wild counterparts (Allan et al., 2018; Cabana et al., 2019). However, the impacts of diet change on gut bacteria remains to be investigated during animal relocation.

Few studies have shown how host-associated microbiota vary between captivity and relocation into a natural habitat and mainly focused on the impacts of place of birth and the immediate environmental exposure (Metcalf et al., 2017; Chong et al.,2019; Schmidt et al., 2019; Yao et al., 2019). Even less studies looked at the effect of captive diet on the gut microbiome during animal relocation (Martinez-Mota et al., 2019). Overall, animals born in captivity have lower α-diversity and more differences in microbial communities compared to animals born in nature reserves or in the wild (Metcalf et al., 2017). Furthermore, deer mice (*Peromyscus maniculatus*) born in captivity and later released had gut microbial communities closer to their wild counterparts compared to animals that stayed in captivity (Schmidt et al., 2019). Since the literature gap of the effects on gut bacteria of captive diet during relocation remains unaddressed, I focused on the impact of captive diet for the gut bacteria during the relocation process in a generalist species with an omnivorous diet. I hypothesized that diet during captivity will affect the gut microbiota of the host during the relocation process and can maximize the reintroduction success of an animal back into its natural habitat. I predict that a wild-like and non-processed diet in captivity would foster the recovery of a wild-like microbiota after the animal is relocated in their natural habitat, compared to a standard captive diet composed of pellets. Therefore, captive diets reflecting a wild diet could have benefits associated with improved degradation of food items by microbes, echoing higher microbial diversity in the gut of mice under a wild-like diet compared to a standard and processed diet in captivity. This study was conducted on the gut bacterial communities of the white-footed mouse (*Peromyscus leucopus*), an omnivorous rodent native to Ontario (Canada) that feeds primarily on insects, seeds, nuts and fruits, just like its closely related species, *Peromyscus maniculatus* (Wolff et al., 1985). *Peromyscus leucopus* does not face major threats of extinction, but its large distribution, short generation time and high capture-recapture rate in general make it an adequate model to study gut microbiota variation across a short period of time to simulate a captive breeding program for reintroduction purposes.

6.3 Materials and Methods

6.3.1 Sample collection

All methods were approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University and by the Toronto Zoo Animal Care and Research Committee (ACRC) under the reference 2018-05-02. White-footed mice (*Peromyscus leucopus*) were trapped on the grounds of the Toronto Zoo (ON, Canada) using Longworth traps. Trapping occurred 5 nights a week during the breeding season of June to mid-September 2018. Each mouse was identified with unique numerical tags (National Brand and Tag co., Newport, KY, USA) and weighed. Wild juveniles were detected by the color of their fur (grey) and their weight (<15 g) and were excluded from the study. Fecal samples were collected directly from the animal using flamed and 70% ethanol-sterilized tweezers and stored in sterile micro-centrifuge tubes in a -20 °C freezer until DNA extraction.

6.3.2 Experimental design

Pregnant dams were brought into an animal holding unit within the Wildlife Health Centre of the Toronto Zoo (ON, Canada) in June and July. Twenty-one days after parturition, the offspring were separated from their dam and housed in individual cages ($\bar{x}$ = 4, 5 juveniles/litter). Animals were placed in individual disposable plastic cages (37.3 x 23.4 x 14.0 cm; Innovive, San Diego, CA, USA) with cut straw from wheat, nesting material (Ancare Nestlets), and PVC tubes for environmental enrichment. Food and water were provided *ad libitum*. The mothers were fed with standard rodent chow. To limit bias from any maternal effects, one half of each litter was given a control diet, becoming part of the Captive Control group (CC) and the other half received the treatment diet, belonging to the Captive Treatment group (CT). For all the groups in this experimental design, the first letter corresponds to the external environment of the mouse at the time of fecal collection and the second letter to the diet they received during their time in captivity (Fig. 1). The control group received the same diet as their mothers, and the treatment group received a diet composed of sunflower seeds, diced apples, crushed walnuts, mealworms, and crushed corn in equal proportions. Each animal received its respective diet and was kept in these conditions for 30 days until they reached sexual maturity. Fecal samples were collected eight days and one day prior to release for each individual (Tab. 1). Those samples represent the Captive Control (CC) and Captive Treatment (CT) study groups. All offspring were then released at one of three locations on the grounds of the Toronto Zoo. Fecal samples were collected from all wild adults trapped in this period at least twice, seven days apart (W for Wild experimental group; Tab. 1). Dams that gave birth in captivity were released but were excluded from this study group. For each recapture of released offspring, becoming respectively the Relocated Control group (RC) and the Relocated Treatment group (RT) depending on their diet in captivity, fecal samples were collected opportunistically, and all were included in the sampling design. It occurred that some relocated mice were never recaptured, and others were recaptured multiple times. Some wild and relocated mice experienced botfly *Cuterebra* sp. infection

during the sampling period. It is characterized by subcutaneous swelling around the genital area (warble) of the mice and the movement of the infectious larvae in this swelling. The presence or absence of infection was considered in the sampling and analysis of the data.

6.3.3 DNA extraction and sequencing

Gene amplicon sequencing was used to study the bacterial communities. DNA extractions from the fecal samples collected were conducted using the Stool DNA Isolation kit (Norgen Biotek Corp, Thorold, ON, Canada) following the manufacturer's instructions. Two blank extractions were made to control for contamination during the extraction process. After DNA extraction, the targeted gene for taxonomic affiliation (16S rRNA gene) was amplified through Polymerase Chain Reactions (PCRs). The library preparation and sequencing were performed by Metagenombio Inc. (Toronto, ON, Canada), as well as the demultiplexing of the sequence reads. Using their designated library protocol, 2 x 300bp paired-end sequencing was completed using broad bacterial primers of the region V4 of the 16S rRNA gene (515F-806R) using an Illumina MiSeq platform (Illumina Biotechnology Co., San Diego, USA).

6.3.4 Bioinformatics

The quality controls of the already demultiplexed paired-end sequence reads were performed through the software FastQC (Andrews, 2010). Sequence reads denoising and Amplicon Sequence Variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al., 2018; v. 2019.1), using the DADA2 pipeline (Callahan et al., 2016; Callahan et al., 2017). ASVs - or also referred to as bacterial phylotypes - were then screened to the 97% 16S rRNA gene full-length reference sequences from the Silva v.132 database (Pruesse et al., 2007) for taxonomical association using the VSEARCH classifier implemented in QIIME2 (Bokulich et al., 2018). Sequence alignment and phylogeny building were conducted in QIIME2 for the construction of a generalized Unifrac distance matrix (α =0.5; Chen et al., 2012). The Cumulative Sum Scaling (CSS) method was used to normalize the data using the *metagenomeSeq* package (Paulson et al., 2013) in R (R version 3.5.2, R Core Team, 2018). It can decrease the fold-difference in sampling depth and avoid the rarefying of counts (Paulson et al., 2013; McMurdie & Holmes, 2014; Weiss et al., 2017).

6.3.5 Statistical analysis for α-diversity of gut bacteria between study groups

All statistical analyses were conducted in R (R version 3.5.2, R Core Team, 2018) using the *phyloseq* (McMurdie & Holmes, 2013) and *microbiome* packages (Lahti et al., 2017) for manipulation of data. Fisher's Diversity index and Simpson evenness index of the phylotypes in each sample were used as metrics to measure the α-diversity of gut bacteria between

samples. Differences in the indexes according to study group, sex, date, infection status, place of birth and interactions were analyzed using linear mixed models with a restricted maximum likelihood estimation approach with mouse ID and dam ID as random factors, using the lmer function in the *lme4* package in R. The study group variable was first considered as two distinct factors: diet and environment. If the two factors and the interaction between the two had a significant effect on the variable, they were combined as "study group". ANOVAs with Satterthwaite's method were run on these models, as well as post-hoc Tukey method for p-value adjustments were conducted to investigate differences between groups. Normality of residuals was validated using the Shapiro-Wilk test. The significance cut-off was set to p-value<0.05 for each test.

6.3.6 Statistical analysis for β-diversity of gut bacteria between study groups and differential abundance

A generalized Unifrac distance matrix between samples (Chen et al., 2012) was used to investigate differences in gut microbial communities between groups, sex, for maternal effect and *Cuterebra* infection. This metric takes into account the differences in phylogenetic distance and abundance of each bacterial communities between samples, pairwise. A PERMANOVA model adonis from the *vegan* package was constructed with 9999 permutations with reported F, R2 and p-values, to determine if there were significant differences between the study groups (Oksanen et al., 2019). Mouse identification tag was used as stratification to account for repeated measures and the model included the sex of the individual, the dam ID, infection status, interactions between those factors, and the study groups in a similar way as the α-diversity analysis. A Detrended Correspondence Analysis (DCA) was conducted to detect the gradual structure in the samples. As multiple samples come from the same individual across time and different environments, the transition from captivity to the wild can be considered as a gradient. The fact that the correspondence analysis is detrended improves the dispersion of point in the ordination of the samples by generalized Unifrac distances and removes the arch effect. Finally, a minimum spanning tree was constructed using the *phyloseqGraphTest* package (Callahan et al., 2016).

The differential abundance analysis was conducted on the ASVs that were present in more than 5% of all the samples and that had a relative abundance of more than 5% among all taxa. It corresponds to the core microbiota of the dataset, represented by 653 phylotypes. The phylotype abundance analysis was made using the *DESeq2* package (Love et al., 2014), using a negative binomial Wald test to test significance in contrast between each study group. Only phylotypes with a significance level (α) below 0.001 after False Discovery Rates (FDR) corrections were considered using the Benjamin-Hochberg method.

6.4 Results

A total of 874,824 sequences of 3,206 bacterial phylotypes (or ASVs) were identified from the 109 samples after the removal of the features present in the two blank samples to avoid DNA extraction bias (mean sequences by samples: 7,043; min: 4,280; max: 14,927). In total, 36 mice were included in the study (*n*=18 W; *n*=10 CC; *n*=8 CT; *n*=5 RC; *n*= 6 RT) and 109 fecal samples were obtained from those mice (*n*=36 W; *n*=20 CC; *n*=16 CT; *n*=8 RC; *n*=29 RT). The low number of mice in the RC and RT groups is due to the fact that the other mice released were not recaptured after relocation (Tab. 1).

6.4.1 **A**-diversity of gut bacteria between study groups according to the host's birthplace

Some relocated and wild animals were sampled during a *Cuterebra* infection (*n*=26 infected; *n*=79 non-infected; Tab. 1), but they were not treated separately in the statistical analysis as it explained 1% of the community variation (anova: F=0.0171; p=0.896237). There was a significant difference in terms of phylotype evenness between mice born in captivity and in the wild (Simpson's Evenness Index: F=2.785; p=0.01877; Fig. 2A), so that mice born in captivity carry gut communities less uniform in phylotype abundance. The interaction of host sex and study group also had a significant impact on the gut bacterial phylotype richness (Fisher's Index: F=6.2087; p=0.006176; Fig. 2B). Male mice from the CC group had significantly higher gut bacteria phylotype richness compared to females from the same group (Tukey: F=4.4974; p=0.031458) or compared to males from the wild (Tukey: F=4.4974; p=0.03992457; Fig. 2B).

From the fecal samples collected, the gut microbial communities of wild mice contained 834 unique phylotypes (5.3% of their gut bacteriome) which is more than captive mice (586, 3%) and relocated animals (525, 1.5%). Relocated and wild mice had 250 common bacterial phylotypes in their gut which represent a higher proportion (8.8%) than the 238 phylotypes common between relocated and captive (7.3%), and between wild and captive individuals (141, 2.7%). Overall, the three groups had 573 phylotypes in common (71.3%). Similar proportions were found between gut bacteria of wild mice and mice that had the control and treatment diets.

6.4.2 B-diversity of gut bacteria between study groups

As expected in mammal gastrointestinal tracts, all samples were dominated by the *Firmicutes* and *Bacteroidetes* phyla (Fig. 3A; McKenzie et al., 2017). Males and females were not treated separately in subsequent statistical analyses. The gut bacterial community composition of male and female mice considered in the study (adonis: F=3.6162; R^2=0.02795; p=0.23676) were

not significantly different and all explained around 3% of the variation. *Cuterebra* infection did not have a significant effect on gut community composition as it explained 1% of the community variation (adonis: F=1.4469; R^2=0.01060; p=0.269730), neither was litter affiliation (adonis: F=3.9089; R^2=0.09062; p=0.12887).

Fecal microbiota differed more in composition between captive and wild mice than between relocated and wild mice (Fig. 3; Fig. 5; Fig. 1 Supp.; adonis: F=2.9232; R^2=0.08742; p=0.000999). Detrended Component Analysis (DCA) on generalized Unifrac distances demonstrated differences of microbiota between the study groups (Fig. 4A). The ordination plot shows that microbiota from captive and wild mice are more distant to each other, compared to relocated (RC and RC) and W mice. A minimum spanning tree generated using a generalized Unifrac distance matrix also shows that microbiota from wild mice are closer to microbiota of relocated animals compared to the ones from captive mice (Fig. 5). Study groups tend to aggregate together, and it was mainly driven by different abundances of taxa in the families *Lachnospiraceae*, *Muribaculaceae* for example, as shown by a split biplot (Fig. 4; Fig. 5). When running the analysis on samples coming from CC and CT mice only, differences among the samples were explained by the diet but also through litter affiliation (Fig.5; adonis: F=1.4141; R^2=0.01170; p=0.037962).

When considering the diets in the relocated and captive groups, the microbiota from RT mice was overall more closely related to the W mice microbiota than the RC mice microbiota (adonis: F=2.9232; R^2=0.08742; p=0.000999). The microbiota from mice of the CT group was then more closely related to microbiota from mice of relocated groups (RC and RT) and W compared to the CC group (Fig. 5).

6.4.3 Differential abundance among groups

The assessment of the differential abundance of bacterial phylotypes using a negative binomial Wald test was conducted on the core microbiota of 653 phylotypes. From those, 62 from four phyla varied significantly among the study groups (*Bacteroidetes*, *Espilonbacteraeota*, *Firmicutes* and *Tenericutes*; Fig. 6; Tab. 2). 60% and 22% of the phylotypes with differential enrichment across groups respectively belonged to the *Lachnospiraceae* and *Muribaculaceae* families. Mice from the CC and RC groups had the greatest loss in abundance in gut phylotypes compared to the other study groups (Tab. 2; respectively 9 for CC and 14 for RC). Overall, the RT group was the only group that had significant phylotypes enriched and in common with gut communities from W mice compared to the other study groups (Fig. 6; Tab. 2).

6.5 Discussion

6.5.1 **A**-diversity of gut bacteria between study groups

I compared the gut microbiota of mice in captivity under different diets, after relocation, and in the wild. The structures of the gut microbial communities in terms of phylotype richness were similar among study groups, with significantly higher phylotype richness only observed in the gut microbial communities of male CC mice compared to females and to wild males. This result is not common on gut microbiome studies in wildlife, and explains no or little variation (Wasimuddin et al., 2017; Schmidt et al., 2019). In that case, captivity could have a sex-specific effect on the gut microbiota of *Peromyscus leucopus*. However, I found no apparent differences in community composition on beta diversity analyses, these results could thus be an artifact from low sample sizes.

The structure of the microbiota in terms of evenness is more uniform in wild-born mice than captive-born mice that have more disparate microbial communities. Similar results were found in studies including the place of birth as a factor of variation in gut microbiota for horses and deer mice (Metcalf et al., 2017; Schmidt et al., 2019). Kohl and Dearing (2014) also observed that evenness decreased with time spent in captivity in desert woodrats. It has been hypothesized that this difference could be due to lasting founder effects of colonization of the gut by microbes during the early life of the host. The natural habitat would be the source of more diverse bacterial phylotypes (interactions with more species, diverse substrates and diets, seasonality, and no antibiotic treatments) compared to captivity. However, the opposite trend was observed in Andean bears and red pandas (Borbón-García et al., 2017; Kong et al., 2014). Host diet, phylogenetics and position in trophic networks could thus be important factors to consider. Overall, the evenness in bacterial communities can affect the subsequent response to disturbances and is known as the insurance hypothesis (Wittebolle et al., 2009), suggesting that place of birth may have on impact on host survival from the gut microbiome aspect. However, it is worth mentioning that differences in diversity indexes between wild and captive mice might be due to the fact that there is no knowledge about relatedness between animals of the W group, whereas captive-born animals come from a handful of litters that can have an impact on the gut microbiota (Spor et al., 2011).

6.5.2 B-diversity of gut bacteria between study groups and differential abundance among study groups

From the β-diversity analysis, I observed that RT and RC individuals were the closest to their wild counterparts in terms of microbial structure and composition than CC and CT animals (Fig. 4&5). This would imply that the immediate environment has a strong effect on gut microbiota composition. Once the individuals are relocated in their natural habitat, the environment becomes the main source for microbes' horizontal acquisition, in both external

exposure but in diet as well (Colston, 2017). Therefore, the captive diet seems to have a smaller impact compared to external exposure but appears to have lasting effects on the gut microbiota, since it influences its composition and structure even one month after relocation (Fig. 6).

The reduced influence of the diet compared to the external environment is also reflected in specific phylotype abundances. Similar to Schmidt et al. (2019), the *Lachnospiraceae* family is differently distributed between the gut microbiota of captive, wild and relocated animals. Although they are present in all groups, *Lachnospiraceae* phylotypes are mostly enriched in W and RT groups rather than CC, CT and RC groups. Maurice et al. (2015) examined the variation of *Lachnospiraceae* in wild *Peromyscus* species. They hypothesized that seasonal variation in the abundance of this taxon is linked to a diet shift from insects to seeds in mid-summer because these bacterial groups support the degradation of complex plant materials. These taxa seem to play a role in the degradation of butyrate during fibre degradation that promotes colonocyte health, immune defense and anti-inflammatory action, reducing the risk of developing metabolic disorders that are a growing concern in captive populations (Meehan & Beiko, 2014; Vijay-Kumar et al., 2010). However in our study, the abundance of the *Lachnospiraceae* family is stable between the study groups but, at a lower taxonomical level, genera abundances within this family seem to differ. The *Lachnospiraceae NK4A136 group* had the highest variation between groups: increasing in RT and W mice and decreasing in the other groups. Not much is known of this genus, but it is associated with the digestive tract of mammals, using carbohydrates and producing short-chain fatty acids (Meehan & Beiko, 2014). Further studies targeting the *Lachnospiraceae* groups would be needed to investigate to which extent there is variation in these taxa between the study groups and their role in the mouse gut.

However, the Lachnospiraceae taxa seemed to be absent or reduced in abundance in the gut of the RC mice and those hosts may have loss the beneficial microbial function linked to these taxa. The fact that the RC group had the highest number of phylotype abundance reduction is another indicator that standardized pellets might not be adequate for animal relocation from the gut bacteria perspective. Few studies to date already advocate for a transitional period between captivity and relocation to foster reintroduction success (Yao et al., 2019), and our results recommend similar practices for generalist species like Peromyscus leucopus. I encourage the production of similar work on hosts with different ecological niches and gut physiology among different taxa, such as Martinez-Mota et al. (2019) that demonstrated similar results than this study on specialist woodrats.

The *Muribaculaceae* family from the *Bacteroidetes* phylum also followed a similar pattern in terms of variation in abundance: it decreased for some phylotypes in all groups, mostly in the

gut of RC mice, and only increased in some phylotypes in the gut of W individuals. A targeted analysis on this taxon would be necessary to understand which exact phylotype varies in abundance. This family, previously named S24-7, is a dominant bacterial group from the mouse gut. It takes part in the degradation of carbohydrates and produces enzymes involved in the degradation of plant glycans like pectin (Ormerod et al., 2016). Pectin is highly present in apples so it could explain the presence of this bacterial group in treatment individuals, but there is no particular enrichment of this taxon in the gut of the CT and RT group. This could be explained by the presence of other fibrous food items in the wild mice diet and therefore encourage the optimization of the treatment diet.

Overall, this study reports complementary results advocating that captivity does have an impact on the gut microbial communities of generalist rodents like *Peromyscus leucopus* after relocation in their natural habitat. Moreover, altered diets in captivity contribute to those effects. Analogous to Sonnenburg et al., (2016), mice subjected to standard low-fiber diet recovered less microbiota diversity than mice fed with a high-fiber, less processed diet. However, the diversity was not in terms of total phylotype richness but in terms of common bacterial groups with the wild 'original' state of the microbiota. The generalization that captivity induces an imbalanced microbiota linked to negative effects on the host should be considered with caution, because it can depend on the taxonomy and ecology of the host, as demonstrated by Greene et al. (2019) and Frankel et al. (2019).

It is also worth mentioning that across the studies comparing the gut microbiota of captive and wild animals; some enclosures allow access to open areas, social interaction, and enrichments that favor exposure to the natural habitat of the species (Clayton et al., 2018; Greene et al., 2019). This could shift the gut microbiota of these animals towards a wild-like state, however this was not the case in our study and microbiota variation between CC, CT, RC and RT mice could only be due to diet. Our study demonstrates that for the relocation of generalist rodents, it is not only a matter of captivity itself and external exposure, but also about diet manipulation. Even if the treatment diet might not reflect all the aspects and components of a wild diet for *P. leucopus* and could lead to nutrient deficiency over a long period of time, it is more adequate than standardized pellets for supporting microbiota composition of mice after relocation. Further work on gene expression in the microbiota and on the host's survival should be undertaken to understand the long-term effects of diet and microbiota variation once an animal is relocated.

It is worth mentioning that the recapture rates between the two relocated mice groups were different. The 75% of RT mice released were recaptured 29 times compared to only 8 times for the 50% of released RC group (Tab. 1). This could be a survival rate indicator, but other ecological factors such as dispersal and predation need to be considered. White-footed mice

are the prey of many animals such as the eastern screech-owl (*Megascops asio*) that was seen on site, and the persistent presence of raccoons (*Procyon lotor*) that disrupted traps and predated on mice (personal observation) might also account for the low recapture rate of RC mice. One explanation could be that RC mice have been more predated than RT mice because of microbiota induced behavior (Enzewa et al., 2012), but further studies and monitoring would need to be undertaken. Finally, no significant results were found in gut microbiota variation due to botfly infection of *Cuterebra* sp. Even if the high prevalence of this infection in *Peromyscus* species has been reported, these parasites have been linked to little effect on host population densities or fitness in general (Slansky, 2007). Our results confirm this trend from the microbiota perspective.

This study simulated how captive breeding programs can impact the relocation process of animals under *ex situ* conservation actions. I demonstrated that captive diet has an impact on the microbiota of a generalist host, even after relocation to a natural habitat. As the gut microbiota takes part in many aspects of an animal's biology, survival and reproductive success, one should consider the microbiota aspect as well as the host's nutrition for the development of diets in captive settings. Researchers should continue to study the effect of captivity on the reintroduction process of endangered species at different scale levels: ecosystem, population, individual and microbiota, and integrate them into management practices.

6.6 References

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6.7 Tables and figures

(A)

Mouse ID	Sex	Dam ID	Diet	*Cuterebra* sp. (after relocation)	Samples collected in captivity	Samples collected after relocation
691	M	D	Control	Presence	2	4
999	F	A	Control	Absence	2	1
1353	F	B	Control	Absence	2	1
1995	M	B	Control	N/A	2	0
6110	F	A	Control	N/A	2	0
7221	F	C	Control	Absence	2	1
7471	M	C	Control	Absence	2	1
8022	M	C	Control	N/A	2	0
8624	M	D	Control	N/A	2	0
54100	F	A	Control	N/A	2	0
Total by group					Captive Control: 20	Relocated Control: 8
496	M	D	Treatment	Presence	2	8
1407	F	A	Treatment	N/A	2	0
6057	F	A	Treatment	Absence	2	1
7631	F	C	Treatment	Absence	2	4
7975	M	C	Treatment	Absence	2	9
8362	F	D	Treatment	Absence	2	1
8887	M	C	Treatment	Presence	2	6
9798	M	D	Treatment	N/A	2	0
Total by group					Captive Treatment:16	Relocated Treatment29

(B)

Mouse ID	Sex	*Cuterebra* sp.	Samples collected
52	M	Presence	2
58	M	Presence	2
59	F	Presence	2
65	M	Presence	2
89	F	Presence	2
92	M	Presence	2
93	F	Presence	2
124	F	Presence	2
146	M	Absence	2
1	M	Absence	2
3	M	Absence	2
18	M	Absence	2
51	F	Absence	2
73	F	Absence	2
82	F	Absence	2
Total			Wild: 36

Table 1 – Individual mice and related samples involved in the study. (A) Mice and related samples that were born in captivity and were under the two different diets. Once relocated in the wild, some mice were not recaptured and others have been recaptured more than once. All samples collected from the recaptured mice have been included in the study. (B) Mice and related samples collected from the wild that never experienced captivity. Some had botfly infection and its effect has been taken into account in the later analysis.

			Captive Control		Captive Treatment		Captive Control & Captive Treatment		Relocated Control		Captive Control & Relocated Control		Relocated Control & Relocated Treatment		Relocated Treatment		Relocated Treatment & Wild		Wild		
Phylum	**Family**	**Genus**	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Reduced	Enriched	Total
Bacteroidetes	**Muribaculaceae**	uncultured bacterium	1		3		1		5				1		1					2	14
	Prevotellaceae	Non assigned																		1	1
		Prevotellaceae UCG-004	1																		1
	Rikenellaceae	Alistipes	2								1										3
	Rs-E47 termite group	unknown													1						1
Epsilonbacteraeota	**Helicobacteraceae**	Helicobacter							1												1
Firmicutes	**Lachnospiraceae**	[Eubacterium] xylanophilum group					1		1											1	3
		Coprococcus 2	1				1														2
		Lachnospiraceae NK4A136 group	1		2		3		4		3							4	2	2	21
		Lachnospiraceae UCG-001			1				1												2
		Roseburia							1												1
		unknown	2				2		1					1	1					1	8
	Ruminococcaceae	uncultured	1																		1
	Staphylococcaceae	Staphylococcus																	2		2
Tenericutes	**Mycoplasmataceae**	Ureaplasma																		1	1
Total			9	0	6	0	8	0	14	0	4	0	1	1	3	0	0	4	4	8	62

Table 2 – Summary of significantly enriched phylotypes among the study groups from the DESEq2 analysis. For each mice group, a number of unique phylotypes is reduced or enriched in abundance compared to all the other groups, and belong to the different taxa on the left. Some phylotypes were also common in two groups compared to the others.

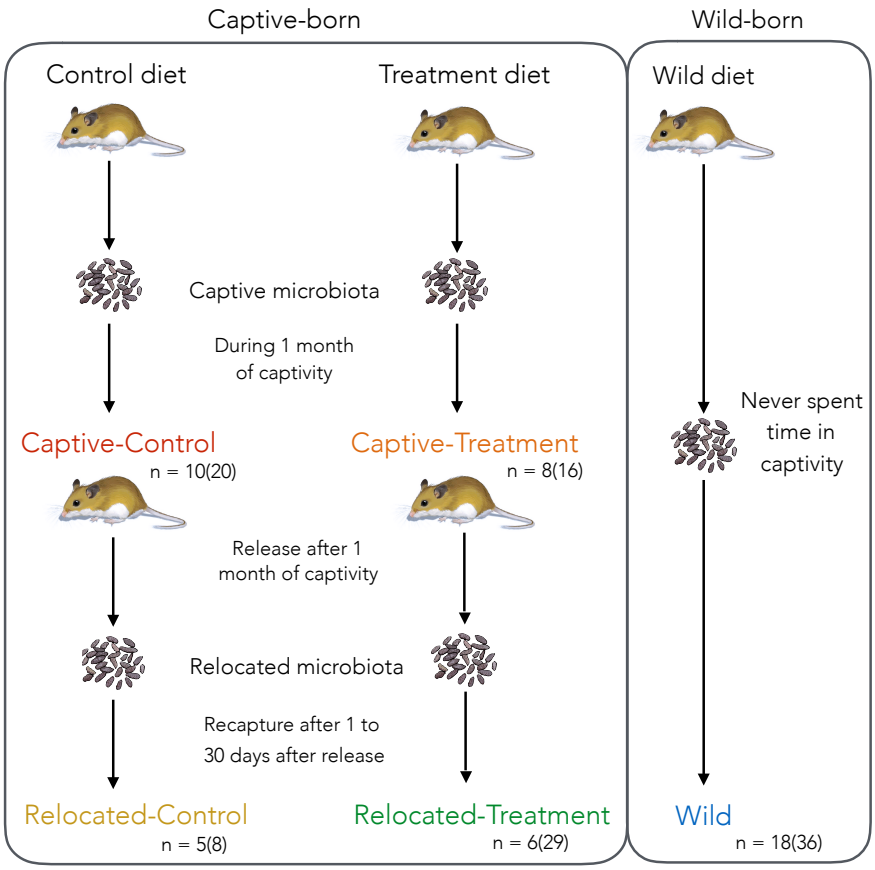

Figure 1 – Experimental design for study conditions and fecal collection. Each color represents the different mice groups, with *n* the number of individuals and in brackets the number of fecal samples overall.

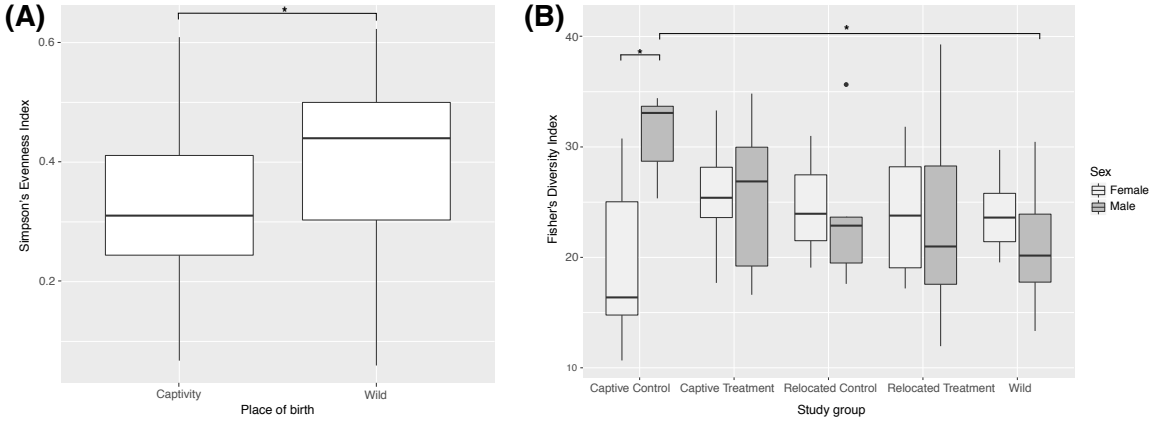

Figure 2 – Boxplots representing changes in (A) Simpson's Evenness Index variation of the gut microbiota depending on the place of birth of the host , and of (B) Fisher's diversity index of gut microbiota among the different study groups and according to the sex of the host. * represents the p-value meeting the standard cutoff $p<0.05$.

Figure 3 – Compared relative abundance of bacterial taxa for each study group of mice in the study (Taxa showing less than 0.1% of relative abundance were not included). In each group, samples are sorted by mouse individual and by date. Stacked barplots showing the relative abundance at the (A) phylum and (B) family levels for gut bacteria among the study conditions.

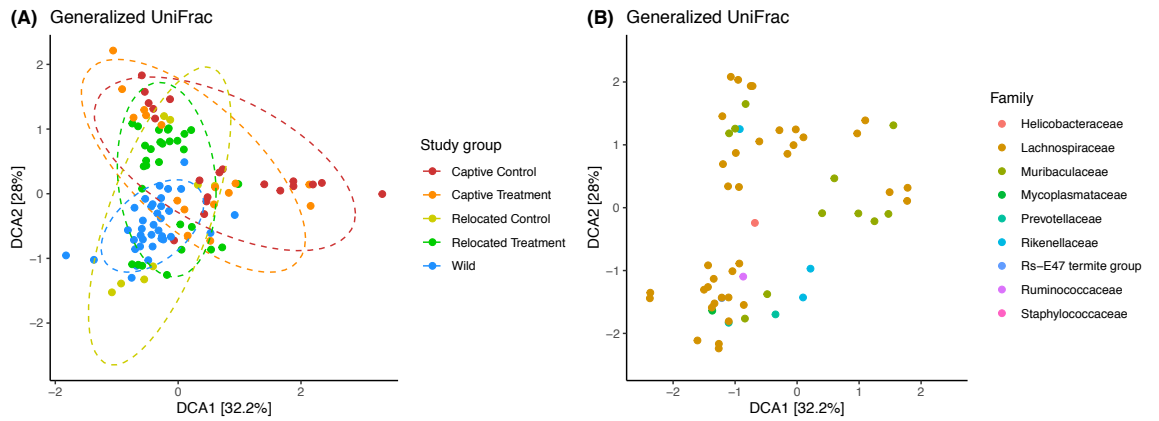

Figure 4 – The Detrended Component Analysis (DCA) ordination method was used on generalized Unifrac distances between samples to visualize differences of microbiota in the experimental groups. (A) Microbiota of mice from the different experimental groups with 90% confidence limit ellipses. (B) Plot showing the 62 significant bacterial phylotypes that

were differentially abundant in the experimental groups after DESeq2-based analysis, according to the DCA ordination method.

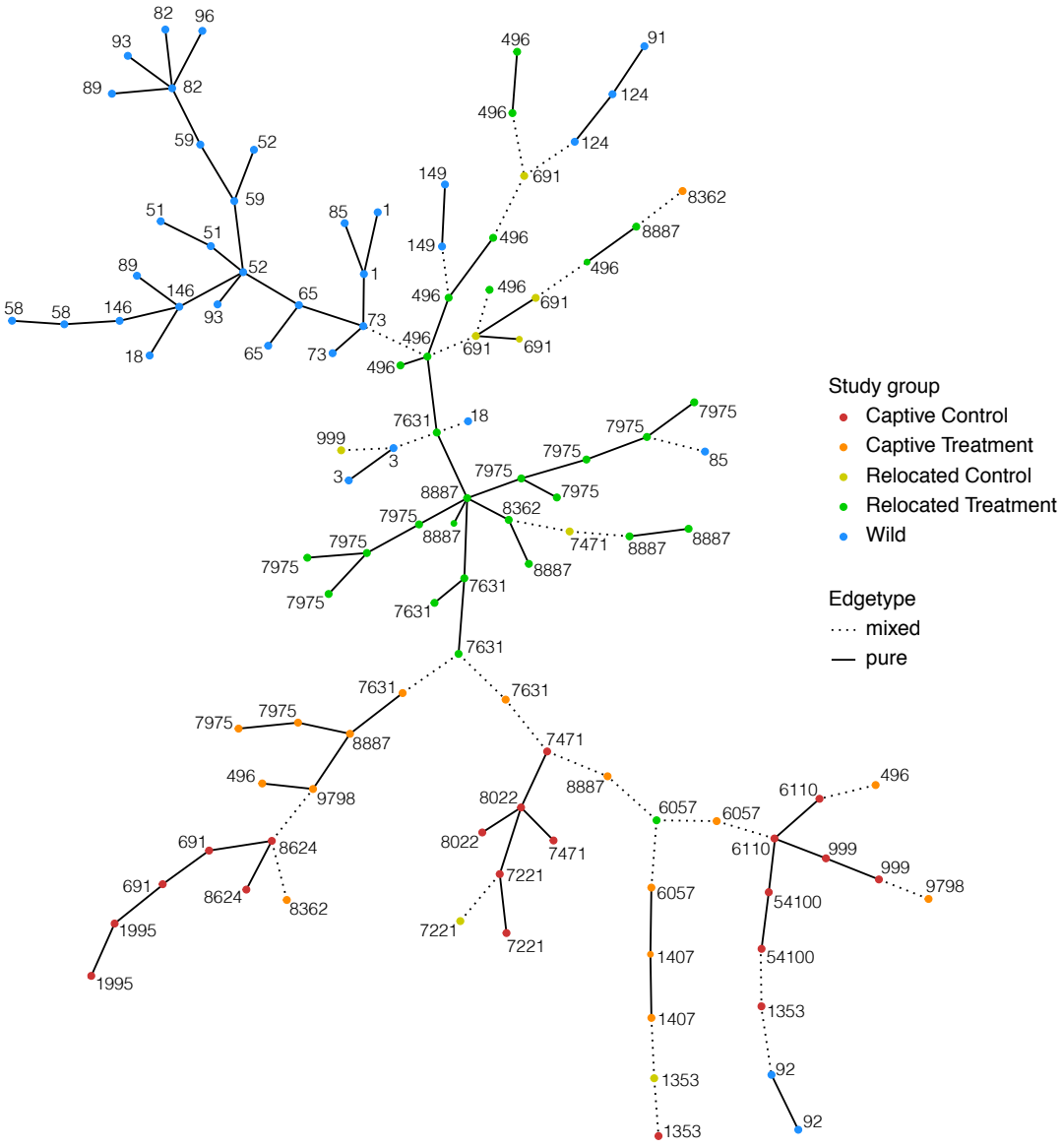

Figure 5 – Minimum spanning tree of samples based on generalized Unifrac distances on all phylotypes. From 9999 permutations, this tree was obtained with 73 pure edges on 104 with permutation p-value < 0.0001. Colors represent the study groups and each sample is named after the mouse identification tag.

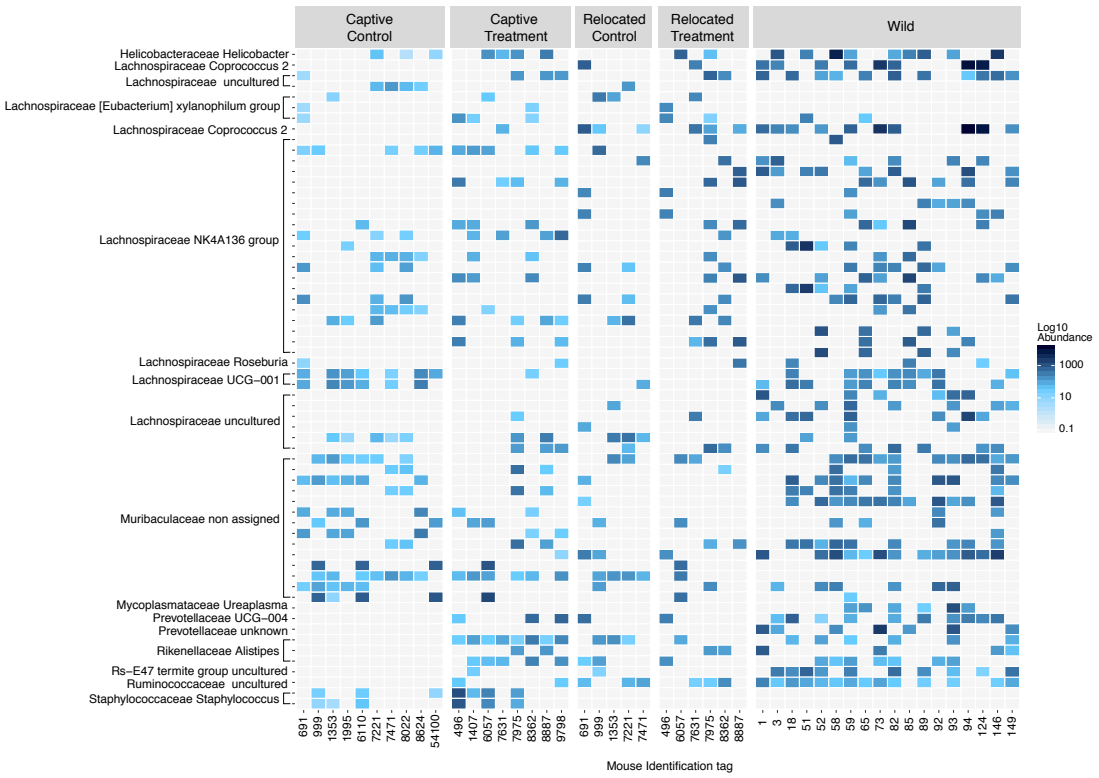

Figure 6 – Heatmap representing the results of the differential abundance analysis. Samples on the x-axis are grouped by mouse identification tag and by study group. The different colors represent the abundance on a log10 scale of each significantly enriched phylotype (median from all samples by mouse). Each phylotype on the y-axis is named by family and genus.

6.8 Supplementary materials

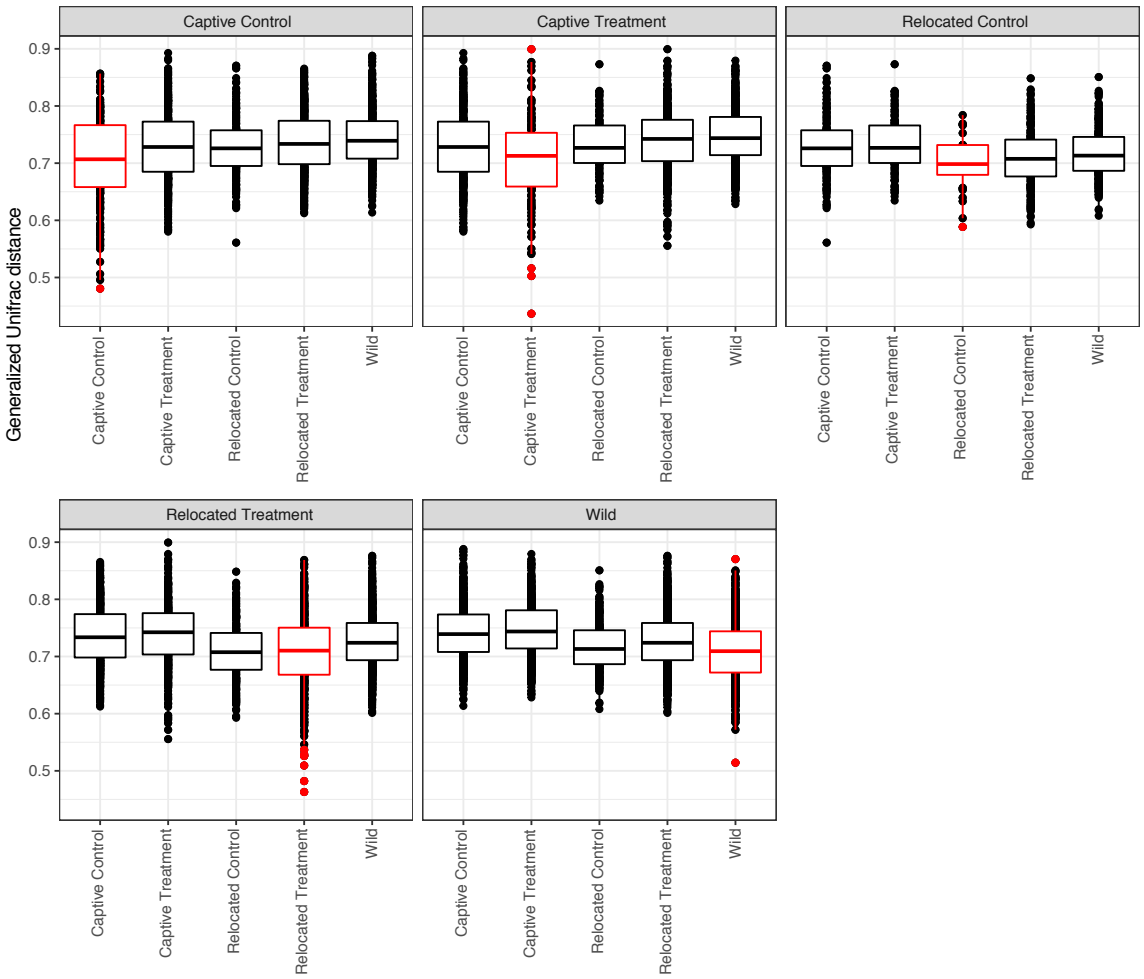

Figure S1 – Generalized Unifrac distance comparison boxplots of all samples between and within study groups. The boxplot is red represent the beta dispersion within the study group and in black of the said study groups between all the other groups.

Chapter 7. General conclusions

7.1 Detangling drivers of variations in GMC

The present PhD study investigated the different drivers of variation in the gut microbial community that can impact captive breeding programs for species conservation. By using white-footed mice, Vancouver Island marmots, American and European minks, I explored the complex dynamics of gut bacteria, host environment, and biology at multiple levels. I postulated that the composition of the gut microbiota is influenced by two broad classes of drivers (Moeller & Ochman, 2013). Heritable drivers may be irreversible, or may only be reversible over multiple host generations, therefore operating on long term scales. I

hypothesized that host genotype and birth location are heritable drivers. Alternatively, immediate factors could induce variation in microbial community composition in a reversible manner and at the individual level of the host. Therefore, I hypothesized that host diet, biology (such as hibernation), and environment (captivity and geography) are immediate short-term factors.

7.2 Host location and diet

When considering gut microbial communities of the Vancouver Island marmot, I observed that microbial diversity was greater in captive animals held in *ex situ* facilities compared to captive and free-ranging individuals on Vancouver Island. This result was especially striking when investigating the microbial variation for marmots transferred between the two captive facilities. There were also a few, non-significant changes in microbial diversity observed between marmots in natural habitats and *in situ* captivity. In this case, I was unable to distinguish the host location and diet, thus I assume these two factors are linked and most variation could be attributed to the supplementation of wild browse from the natural habitat in the diet at the *in situ* facility. However, enhanced gut microbial diversity in free-ranging animals is the common trend compared to captive conspecifics (McKenzie et al., 2017; reviewed in West et al., 2019). I observed a similar increasing trend in microbial diversity for free-ranging animals in both European and American mink as discussed in chapter four. While it is clear that hosting microbial communities can be functionally beneficial or even necessary to an animal (Harrison et al., 2017), the extent of these benefits is still uncertain. I thus hypothesized that for the Vancouver Island marmot, a less diverse, and potentially more specialized, microbial diversity towards amino acid metabolism is reflective of natural, and therefore, inherent gut microbial communities compared to *ex situ* captive microbial communities. Information on the natural diet of the Vancouver Island marmot is still scarce, but it is possible that this herbivore strictly consumes plants endemic to Vancouver Island, fostering a specific gut microbial community (Werner, 2012).

I also found dissimilarities in microbial community composition in the three models (Marmot, mice, and mink) according to host location. In the fourth chapter, host location overshadowed host genotype, as mink of different species had similar microbiota in captivity when compared to free-ranging conspecifics. I also found that two free-ranging populations of mink from the two species were closer in microbial community composition than two mink populations of the same species in natural habitats. The role of diet in these results was not possible to measure, but based on habitat landscape ecology, I hypothesized that habitats where mink had close microbiota composition would offer similar prey types. Both American and European mink prey on small ground-dwelling mammals that are greatly abundant in agricultural landscapes (Krawczyk et al., 2013). A similar explanation concurs with our results

when considering similarities between *in situ* captive and free-ranging Vancouver Island marmots compared to *ex situ* captive conspecifics in the second chapter. Supplementation of wild *in situ* browse to the captive diet could be the bridge between the two populations, considering access to natural substrates is very limited in both captive settings.

Finally, we can confirm the hypothesis that host location as a driver is related to diet when looking at the results from the sixth chapter. While captive-born and relocated mice had similarities in gut microbial communities with wild-born free-ranging mice compared to captive-born mice held in captivity, the mice receiving the treatment diet in captivity (non-processed diet: whole seeds, fruits, nuts and insects) had more similarities in gut microbiota composition than mice fed a control diet (mice chow). Our results suggest that even though the external environment has a strong influence on gut microbial communities, diet in captivity still plays an important role in their dynamics even after numerous days after relocation. Our results are even more striking considering the low number of mice receiving the control diet that were recaptured, potentially indicating either great dispersion or poor survival compared to treatment mice. It is important to note that gut microbial communities in captive-born mice were more similar to wild conspecifics once they were reintroduced than mice maintained in captivity, indicating the strong influence of host environmental exposure despite the impact of diet modification. Community dynamics may differ when considering the diet specialization of the Vancouver Island marmot compared to the more general diet known in the white-footed mouse.

7.3 Hibernation

In the third chapter, I also observed a decrease in microbial diversity for marmots in both facilities before hibernation, a common trend in other species (Carey & Assadi-Porter, 2017; Kurtz et al., 2021). I observed a linear increase of microbial diversity across time upon emergence from hibernation for both groups, as well as differences in composition in both periods according to birthplace. These results support our hypothesis that biological traits such as hibernation are reversible and immediate drivers of gut microbial diversity variation. Numerous immediate drivers can impact gut microbial communities, as I saw in this chapter, whereby hibernation, host location, and birthplace influenced microbial variation. Overall, changes in gut microbial communities over critical periods such as hibernation can have nutritional consequences on the host. Although it was not possible to measure them in this chapter, metagenome prediction through PICRUSt revealed the potential role of microbial genes involved in lipid, glycans and amino acids metabolism, which are key components for survival during hibernation.

7.4 Birthplace

Birthplace was an important driver of gut microbial community variation for the Vancouver Island marmot, where microbial diversity was lower in both captive wild-born and marmots with wild-born parents. The same pattern for host location and diet could apply in the case of the Vancouver Island marmots, where specialized microbes are fostered in the natural habitat over microbial diversity in *ex situ* settings. While birth mode is a key factor shaping early microbial colonization (Selma-Royo et al., 2020), the influence of birthplace remains poorly understood. A recent study on mice explored gut microbial variation in different soil environments during early-life stages (Liu et al., 2021). After changes in housing environments, mice harbored 1/3 of microbial assembly that was significantly linked to prior soil-exposures. Early-life exposure to different environments was also associated with the magnitude of temporal microbiome change due to environmental shift, indicating potential consequences on host development (Liu et al., 2021). While those consequences are unknown in the Vancouver Island marmot, it is worth investigating if early exposure to soil from the natural habitat for captive marmots could be beneficial to foster a "wild-like" microbiota. The sixth chapter also brought some light on the importance of early-life microbial colonization through diet because the diet modification experiment was done from weaning to sexual maturity for white-footed mice. I observed long-lasting variation in gut microbial communities one day or one month after relocation that I believe was influenced by the change diet from captive to wild environments.

7.5 Genotype

In the fourth chapter, I investigated the relationships between gut microbial community assemblage and host genotype between two closely related species of mink. I observed that host genetic relationships were overshadowed by host location and potentially diet. Captive European mink had fewer gut microbial dissimilarities with captive American mink than free-ranging conspecifics. As previously mentioned, diet may have a strong influence in this study system. However, in the fifth chapter I evaluate the role of immunogenetics in the context of captive breeding programs when it comes to gut microbial communities. Because the diet of the European mink populations was similar between the two captive facilites, I observed differences in gut microbial communities that were influenced by both host genetic diversity and divergence. When sexual selection is limited to foster genetic diversity, our results suggest that both MHC genes influence different sets of microbial taxa. The differences between the influences on these microbes may be explained by different management practices between the two populations. Long-term *ex situ* conservation practices can thus modulate gut microbial communities, which may have consequences on the survival of reintroduced animals. To foster host genetic diversity and increase microbial community uniformity, supplementing breeding stocks with free-ranging individuals and limiting the number of

generations in captivity may be viable strategies. The supplementation of free-ranged individuals may be effective because sexual selection is less restrictive.

7.6 Conserving the germs

Biology is undergoing a paradigm shift, where individual phenotypes are seen as a result of complex interactions resulting from the combined expression of the host and associated microbial genomes, leading to the popularization of the holobiont concept (Bordenstein & Theis, 2015). Throughout this PhD study, I assumed that gut microbial communities found in free-ranging animals within their natural habitat was the gold standard to foster for host conservation. However, one can wonder if it is the best practice, and if microbial variations are inevitable and not reversible, considering the drivers investigated when animals are in captive settings. While gut microbiota variations seem inevitable in an individual's lifetime, it is critical to identify which changes are stochastic, without consequences on the host through functional redundancy, and changes that could be detrimental to the host's survival. Investigations in humans have shown how the Anthropocene has altered our microbiome, with industrialized microbiota diverging from ancestral states (Cho & Blaser, 2012). Such changes have been linked to the rise of non-communicable chronic human diseases, such as obesity, insulin resistance, allergies, and irritable bowel syndrome (Sonnenburg & Sonnenburg, 2019). If we transpose industrialization for humans to captive breeding in endangered species, consequences on the species survival, especially during the reintroduction process, can be significant. Since composition variation in microbiota will take place when animals enter captivity, modulating the gut microbiota to its wild ancestral states seems to be an unattainable goal. However, colonization of key taxa for the host's nutrition, reproduction, physiology, and behaviour should be fostered, to potentially promote microbial dynamics for communities to acquire as many key taxa as possible from the natural environment once the animal is reintroduced. Our results shed light on the importance of early life colonization and the potential that diet modification has on microbial modulation to foster potentially key taxa in the gut microbiota when hosts are held in captive settings. Considering these results, it is likely that holding animals in captivity within the distribution area of the species could foster ancestral states, but diet should include as many natural components of the species diet as possible. Additionally, practices where sexual selection can happen should be considered.

Holobiont research is now informative across numerous fields of the life and medical sciences, including aspects of mathematics (bioinformatics, statistics, and modeling), and species conservation is no exception (West et al., 2019). As globalization, climate change, human population expansion, and the natural and unnatural movements of species interact to become a central issue in biological conservation, which itself has become an interdisciplinary

web that embraces the health of ecosystems and people, such as the One Health initiative (Rabozzi et al., 2012). Because even the pathogen concept is questioned today in the light of the microbiome field, the entire wildlife biology domain needs to be redefined to include the microbial world. That is why the concept of holobiont is important, to bring microbiologists to the conservation biology table, fostering new discoveries as we face a new frontier to overcome, and to better understand our natural world.

7.7 References

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