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Multi-omics insights into the mammalian gut microbiota composition and functionality

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Summary

The gastrointestinal tract of mammals is inhabited by a highly complex and extremely heterogeneous microbial community, including bacteria, viruses, archaea, fungi and protozoa, which reaches the highest density in the intestine where microorganisms form the so-called gut microbiota. Despite the wide variety of microorganisms that may colonize the mammalian intestine, in recent decades scientists have paid particular interest to the study of the bacterial component of the intestinal microbial consortium since not only bacteria are by far the most abundant members of the mammalian intestine, but they are also involved in a continuous dialogue with the host ultimately affecting its health. Traditionally, the characterization of this bacterial ecosystem was based on culture-dependent methods which, although allowing biochemical and physiological investigations, do not allow the isolation of all bacterial species colonizing the mammalian intestine, thus failing to identify all players of the intestinal microbial community. However, at the beginning of the current century, the advent of the Next-Generation Sequencing high-throughput techniques have exponentially increased our knowledge concerning the gut microbiota, allowing us to shed light on the taxonomic composition and functional activities performed by the overall intestinal microbial consortium as well as on the dynamics regulating the myriad of microbe-microbe and microbe-host interactions. Specifically, the combination of culture-dependent methods and high-throughput sequencing techniques revealed that millions of years of co-evolution between the intestinal microbial community and its host have contributed to the establishment of various trophic interactions such as mutualistic relationships from which both parties benefit. Indeed, the gut microbiota performs a series of metabolic and physiological functions that influence host biology. However, despite the influence of the intestinal microbiota on host health, most research seems to study and characterize this microbial community exclusively to humans, thus limiting our understanding of the intestinal ecosystem of non-human hosts.

In this context, the aim of this PhD thesis is to explore the taxonomic composition and functional activities performed by the intestinal bacterial community of various mammalian species through the application of a multi-omics approach involving both metagenomics (16S rRNA gene microbial profiling, bifidobacterial ITS microbial profiling and shotgun metagenomics) and metatranscriptomics techniques. Specifically, it aims to explore the taxonomic composition of the gut microbiota of various canine breeds at both genus and bifidobacterial species level and to assess how this bacterial community may be modulated by diet, age and anthropogenic influences. Furthermore, in order to extend our knowledge on the intestinal microbial population of the two principal companion animals for humans, this thesis describes a comparison of the intestinal bacterial composition of dogs and cats at genus and bifidobacterial species levels. In addition, it provides in depth insights into the role that human intervention, due to the domestication process, has played in modulating the gut microbiota of several domesticated-feral mammalian dyads. Finally, a further purpose of this thesis is to evaluate the impact that diet and host digestive system anatomy may have had on the composition, functional activities and gene expression of the intestinal microbial community of a wide spectrum of mammalian hosts distributed along the branches of the tree of life, including herbivores, carnivores, omnivores and piscivores.

Chapter 1

General introduction

A. The gut microbiota: general features of this intricate ecosystem

The trillions of microbial cells that inhabit the human and animal body have promoted the host to a multispecies hybrid 'superorganism' composed of both host and microbial cells interacting with each other in a dynamic and symbiotic equilibrium (Barko *et al.*, 2018). Among the various sites of the mammalian body, the highest microbial densities are reached in the gastrointestinal tract (GIT), especially in the distal intestine, where the number of microbial cells has been estimated to equal the number of host cells in humans (Sender *et al.*, 2016, Sender *et al.*, 2016). Indeed, the mammalian intestine is colonized by one of the most complex, extremely dense and dynamic community of microorganisms in the biosphere, encompassing bacteria, viruses, protozoa, archaea and fungi that are collectively defined as gut microbiota (Turnbaugh *et al.*, 2007). Within this intricate microbial ecosystem, autochthonous or indigenous microorganisms coexist with transient or allochthonous microorganisms that can cross the mammalian GIT as the result of food ingestion or environmental contaminations (Ventura *et al.*, 2009). Consequently, only a relatively small portion of the mammalian gut microbiota is represented by opportunistic pathogens that, by definition, reside unperturbed within the host intestine until an alteration of the gut ecosystem occurs with a consequent disruption of the gut microbiota homeostasis. Despite the wide variety of microorganisms colonizing the mammalian intestine, in recent decades scientists have focused their attention on the study of the intestinal bacterial ecology, since not only bacteria are by far the most abundant representatives of the mammalian gut ecosystem, but they are also involved in an intimate relationship with their host (Hillman *et al.*, 2017). Indeed, millions of years of co-evolution between the mammalian host and its gut bacterial community have favoured the establishment and subsequent consolidation of various trophic interactions, encompassing a mutually beneficial relationship from which both parties benefit (Backhed *et al.*, 2005). While the host offers nourishment and a suitable environment for growth and reproduction of the gut microbial population, the latter, in turn, performs a series of physiological and

metabolic activities, which ultimately exert a marked influence on host health (Thursby & Juge, 2017). Specifically, a balanced gut microbiota is involved in the protection against pathogen colonization and proliferation (Pickard *et al.*, 2017), stimulation and education of the host immune system (Lin & Zhang, 2017), degradation of otherwise non-digestible host- and/or diet-derived complex carbohydrates, maintenance of bowel homeostasis and provision of energy sources to guarantee the basal activity and integrity of the intestinal epithelial barrier and production of various metabolites, including the fermentation end-products, such as Short Chain Fatty Acids (SCFAs), or vitamins (Moens & Veldhoen, 2012, Pickard *et al.*, 2017). However, functional and physiological perturbations of the intestinal barrier as well as alterations in the composition and/or functional activities of the gut microbiota are generally related to the disruption of intestinal homeostasis affecting both the innate and adaptive host immune system, thereby leading to a so-called state of dysbiosis (Tomasello *et al.*, 2016, Pickard *et al.*, 2017).

B. Overview of technical approaches for gut microbiota determination

Despite the wide variety of species of microorganisms and their distribution in multiple ecological niches, a mechanistic understanding of the key roles played by these microorganisms in nature, encompassing those inhabiting the mammalian GIT, is far from being complete (Biteen *et al.*, 2016). Traditionally, the pioneering strategy adopted for the characterization of the intestinal microbial community was based on culture-dependent methods, relying on the use of different culture media for growth and subsequent isolation of microorganisms (Lagier *et al.*, 2012). Although these classic microbiological techniques offer the possibility of performing physiological and biochemical investigations on the isolated bacterial strains as well as enumerating the total viable bacterial load of a given sample, culture-dependent methods suffer from multiple limitations that prevent a comprehensive view of the intestinal microbial community (Turroni *et al.*, 2008). Indeed, culturing is not only time-consuming and laborious, it also requires immediate processing of samples while results are generally affected by both growth media and laboratory conditions, which are unlikely to faithfully reproduce the extremely complex intestinal environment (Bottari *et al.*, 2006, Furrie, 2006). In this context, it has been estimated that only a small fraction of the human and animal intestinal biodiversity can be explored by just employing culturing techniques and pure culture studies (Biteen *et al.*, 2016). The hypothesis that a large proportion of the mammalian intestinal community remains inaccessible with the exclusive use of culturing led to remarkable advances in the molecular field, opening up new research horizons aimed at investigating gut microbiota ecological aspects by applying high-throughput culture-independent approaches, i.e. metagenomics, metatranscriptomics and metabolomics to shed light on the identities, activities and functional roles of the overall intestinal microbial community, thus including uncultivable members (Segata *et al.*, 2013) (Figure 1).

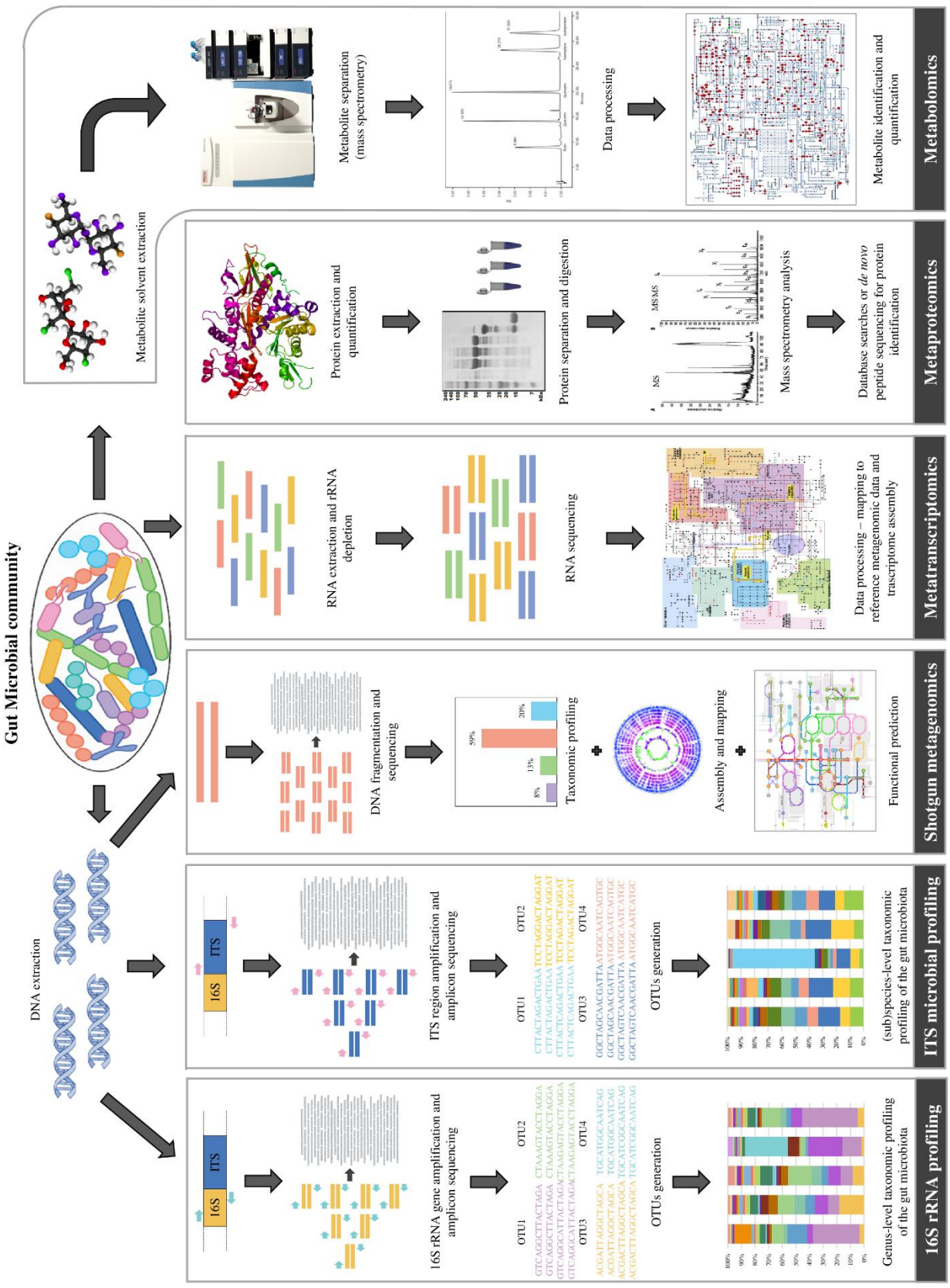

Figure 1. An illustration of the main steps of metagenomics, metratranscriptomics, metaproteomics and metabolomics approaches.

High-throughput methodologies for the investigation of the gut microbiota composition and functionalities

Initiated in the new millennium, the advent of Next-Generation Sequencing (NGS) techniques has completely revolutionized today's biology, giving birth to so-called metagenomics, a new research field that provides an efficient tool to evaluate the taxonomic composition and functional potential of the overall intestinal microbial community (Ventura *et al.*, 2009, Koboldt *et al.*, 2013, Suchodolski, 2016). Among the various NGS high-throughput methodologies currently available, the 16S rRNA gene microbial profiling represents the first culture-independent approach to have been used for the compositional characterization of the gut microbiota (Hamady & Knight, 2009, Honneffer *et al.*, 2014). This method relies on the use of universal primers for a PCR-mediated amplification and subsequent sequencing of a single or multiple hypervariable regions of the 16S rRNA gene, which is a conserved phylogenetic marker gene composed of nine hypervariable regions flanked by highly conserved sequences (Hamady & Knight, 2009, Rajilic-Stojanovic & de Vos, 2014). This reproducible and technically easy procedure, high efficiency in bacterial taxonomy identification, accessible bioinformatic pipelines, and cost-effectiveness have contributed to the election of 16S rRNA gene microbial profiling as the gold standard methodology to catalogue members of the intestinal microbial community (Janda & Abbott, 2007). However, despite the fact that 16S rRNA gene microbial profiling is and continues to represent the most popular method to investigate gut microbial populations, this approach suffers from serious limitations, due to the lack of a universally accepted standardized protocol. Indeed, there is no single procedure for DNA extraction while several primer pairs targeting different 16S rRNA gene hypervariable regions are currently used for the amplification step, thus generating biased bacterial profile-based results characterized by distinct sequencing efficiency and taxonomic discriminatory power (Suzuki & Giovannoni, 1996, Kennedy *et al.*, 2014). As a consequence, since the use of different primers led to an under- or over-representation of specific bacterial taxa of the gut microbiota, conflicting results were reported from

different studies (Klindworth *et al.*, 2013, Milani *et al.*, 2013). Furthermore, the overall 16S rRNA gene length far exceeds the maximum read length achieved by the commonly used NGS platforms, thus failing to provide high taxonomic resolution of complex intestinal microbial consortia (Alcon-Giner *et al.*, 2017). Indeed, the sequencing of a single portion, rather than the full length of the 16S rRNA gene, allows a taxonomic classification down to the genus level only, thus failing to provide a detailed overview of the microbial species colonizing the host gut (Milani *et al.*, 2013).

In order to overcome the limitations imposed by the experimental procedures required for the 16S rRNA gene microbial profiling protocol, novel NGS-based, cutting-edge techniques have been developed to obtain a more detailed and high-resolution image of the intestinal microbial community. For this purpose, the Internally Transcribed Spacer (ITS) sequencing method has been proposed as a valuable tool to accurately profile the bacterial players of the intestinal environment (Milani *et al.*, 2014). Specifically, the ITS region represents a spacer sequence located between the 16S rRNA and 23S rRNA genes within the rRNA locus. Unlike the 16S rRNA gene, the ITS genomic portion is highly variable at the interspecies level, thus representing a valuable genetic marker for in depth assessment into the gut microbiota composition at species or even subspecies level (Milani *et al.*, 2014). An ITS-based approach, known as ITS-bifidobacterial microbial profiling, has recently been proposed to discriminate between closely related bifidobacterial (sub)species and to investigate the ecology of the *Bifidobacterium* genus across the human and animal gut (Milani *et al.*, 2014, Sabbioni *et al.*, 2016, Duranti *et al.*, 2017, Milani *et al.*, 2017). However, this approach is once again dependent on a specific genomic sequence amplification step and it is currently available only for a single microbial genus, thus limiting the all-around species-specific study of the gut microbiota.

In this context, in depth Whole Metagenome Shotgun (WMS) sequencing has been developed as a strategic alternative to overcome the limitations of both the 16S rRNA gene and ITS-bifidobacterial microbial profiling. Bypassing PCR-based amplification and potentially sequencing the total (fragmented) microbial DNA extracted from a specific matrix, the WMS offers remarkably more information about the taxonomic and functional arrangement of a complex microbial environment

when compared to gene- or sequence-specific sequencing approaches (Milani *et al.*, 2017). Firstly, since the overall DNA content of a sample is captured, the WMS method allows detailed identification of the microbial players of a complex environment, including the gut microbiota, at a (sub)species or even strain level (Quince *et al.*, 2017). Secondly, favouring in depth insights into the genomic content of a microbial ecosystem, the WMS technique allows the prediction of the potential functional activities exerted by a specific bacterial taxon (Abubucker *et al.*, 2012, Quince *et al.*, 2017). Indeed, functional classification of shotgun metagenomic data not only allows exploration of the microbial genetic repertoire involved in microbial catabolic and anabolic processes, but it also provides insights into the multitude of other functional aspects characterizing the gut microbiome such as antibiotic resistance, extracellular structures responsible for adhesion or presence of phages (Milani *et al.*, 2017). Furthermore, the WMS have promoted the move forward to shed light on the peculiar functions of the major unexplored portion of the microbial biodiversity, collectively referred to as 'microbial dark matter' (Rinke *et al.*, 2013). Despite the powerful advantages of the WMS, most of large-scale studies continue to prefer the 16S rRNA gene microbial profiling to bypass the high cost required by high-resolution WMS.

Regardless of the method used, the massive scale of generated data from DNA sequencing requires robust processing power and bioinformatics pipelines in order to manage and annotate sequence information. In this context, metagenomic approaches need continuous updating of both the taxonomic and gene reference databases in order to avoid erroneous annotation of metagenomic data (Biteen *et al.*, 2016, Milani *et al.*, 2017).

Furthermore, metagenomics approaches are affected by two other important limitations: they only report relative abundance-based microbial profiling, thus limiting the study of gut microbiota to a qualitative level (Vandeputte *et al.*, 2017) and they only reveal the genetic potential of microorganisms of a given environment, thereby failing in identifying those genes actually expressed (Wang *et al.*, 2015). The first issue has recently been overcome by proposing the combination of a microbial cell enumerating method, i.e. flow cytometry, with metagenomics approaches in order to

achieve quantitative microbial profiling (Vandeputte *et al.*, 2017). The second issue, instead, has been addressed by the development of other complementary high-throughput methodologies such as the NGS-based metatranscriptomics (Poretsky *et al.*, 2009) as well as metaproteomics (Verberkmoes *et al.*, 2009) and metabolomics (Nicholson *et al.*, 2005). Specifically, metatranscriptomics encompasses the massive sequencing of the total mRNA extracted from a given sample in order to elucidate which genes, among the multitude of bacterial genes annotated through a metagenomic analysis, are actually transcribed and therefore expressed. In this context, metatranscriptomics provides a powerful gene expression profile to characterize the active metabolic pathways of a microbial community (Bashiardes *et al.*, 2016). Furthermore, two other -omics approaches, i.e. metaproteomics and metabolomics, have been designed to discover the key metabolic activities of the intestinal microbial community and ultimately reveal the microbe-microbe and microbe-host functional dynamics and interactions (Bashiardes *et al.*, 2016). Specifically, while metaproteomics identifies the arsenal of proteins produced by the intestinal bacterial community, metabolomics is used to unveil the wide variety of metabolites produced by the intestinal microbial consortium (Aw & Fukuda, 2015, Bashiardes *et al.*, 2016).

Culturomics approaches

As mentioned above, culture-dependent methods have in recent decades been progressively replaced by culture-independent approaches in order to obtain a comprehensive overview of the human and animal gut microbiota composition, bypassing the inability to *in vitro* reproduce the intestinal environment, thereby capturing already characterized and isolated microorganisms as well as the unculturable bacterial fraction (Segata *et al.*, 2013). However, this process has caused a substantial knowledge gap between the already cultivated and characterized bacteria inhabiting the intestinal tract and those that have not yet been isolated (Hugon *et al.*, 2015). In this context, it has been demonstrated that only 56% of intestinal bacteria identified through the application of NGS-based approaches possess cultured representatives (Goodman *et al.*, 2011). In order to address this discrepancy, the last decennia have been marked by the re-birth of culturing techniques based on a renewed high-throughput approach, known as 'culturomics' that employs the combination of culture efforts with the identification of isolated bacteria based on their chemical composition through a rapid and cost-effective screening, i.e. MALDI-TOF mass spectrometry (Greub, 2012, Lagier *et al.*, 2015). In this context, the formulation of novel complex growth media and improved anaerobic systems have guaranteed the success of culturomics allowing the cultivation and subsequent isolation and characterization of a substantial number of novel human and animal intestinal microorganisms (Bittar *et al.*, 2014, Hugon *et al.*, 2015, Tandina *et al.*, 2016).

C. Composition and functionalities of the gut microbiota in mammals

Starting from the end of the 19th century when it was first discovered that microorganisms are an integral part of the human and animal intestine and that host health depends on a myriad of microbe-microbe and microbe-host interactions that take place at the site of the intestine, an exponential number of studies have been and currently continues to be directed to unveil the taxonomic composition and functionality of the gut microbiota. Specifically, the advent of the NGS-based high-throughput techniques, at the beginning of the 21st century, have provided new low-cost, powerful and reliable tools to get access to the composition, functionalities and dynamics of the intestinal microbial ecosystem, considerably accelerating the characterization of this intricate community (Behjati & Tarpey, 2013, Koboldt *et al.*, 2013). In this context, several international projects were established, including the NIH Human Microbiome Project (HMP) or the European Union Project on Metagenomics of the Human Intestinal Tract (MetaHIT), aimed at firstly creating a catalogue of the human intestinal microbial players and associated genetic repertoire and ultimately exploring the dynamics governing microbe-host interactions involved in influencing host health (Group *et al.*, 2009, Qin *et al.*, 2010, Arumugam *et al.*, 2011, Human Microbiome Project, 2012, Integrative, 2014). However, these large-scale projects were exclusively human-oriented, thus limiting current knowledge on the intestinal microbial community of non-human hosts. Only in recent years the scientific community has shifted a considerable part of its interest to the characterization of non-human mammalian gut microbiota in order to shed light on the forces that have forged the strategic alliances between a specific mammalian host and its intestinal microbial community in the course of their co-evolution (Ley *et al.*, 2008, Ley *et al.*, 2008, Muegge *et al.*, 2011, Groussin *et al.*, 2017). Indeed, while it is well accepted that the long-lasting process of microbe-host co-evolution is the result of intricate and reciprocal adaptive events responsible for the adaptation of mammals to novel environmental niches and for their dispersal and current global distribution (Moran, 2006, Ley *et al.*,

2008), the factors that have influenced the establishment of this mutualistic relationship have not yet been fully clarified. In this context, apart from the possibility of identifying Firmicutes and Bacteroidetes as the main bacterial taxa characterizing the mammalian gut microbiota followed by Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia, NGS-based techniques applied to fecal samples of a wide range of mammalian species have allowed to identify diet, host phylogeny and physiology as the principal factors responsible for having shaped the mammalian gut microbiome over evolutionary times (Ley *et al.*, 2008, Ley *et al.*, 2008, Muegge *et al.*, 2011, David *et al.*, 2014, Benson, 2015, Groussin *et al.*, 2017).

Beyond the inter-individual differences whereby each mammal apparently possesses its own unique intestinal microbial consortium, the gut microbiota of conspecific hosts is more similar to each other than to that of hosts belonging to different mammalian species (Ley *et al.*, 2008). Based on this observation, the term 'phylosymbiosis' has been recently introduced to describe the eco-evolutionary pattern according to which host evolutionary changes are associated with ecological modulation of their intestinal microbial ecosystem, thus suggesting that host genetic traits that have undergone modifications in the course of evolution not only have generated different mammalian phylogenetic clades but they have also played a relevant role in shaping the mammalian gut community (Ley *et al.*, 2008, Sanders *et al.*, 2014, Brooks *et al.*, 2016). Among others, changes in the physiology of the digestive system across the mammalian branch of the tree of life have driven host-microbe convergent evolution. Adaptations to a plant-based diet have determined an evolutionary breakthrough in mammals that have led to massive radiations resulting in the dissemination of herbivores in most of mammalian lineages with 80% of extant mammals corresponding to herbivorous species (Ley *et al.*, 2008). However, the transition from a protein- to a complex plant-derived carbohydrate-based diet has challenged mammals with the need to find a way to retrieve energy from these new substrates, since mammals lack the necessary enzymes, i.e. glycosyl hydrolases (GHs) and lyases, for degrading plant-associated polysaccharides (Russell & Rychlik, 2001, Ley *et al.*, 2008). To address this challenge, mammalian lineages began to rely on the fermentation processes exerted by fermentative

bacteria that, unlike their mammalian hosts, possess in their genetic heritage the set of genes necessary for carbohydrate breakdown (Ley *et al.*, 2008). Furthermore, to favour these fermentative activities and to retain microbes, mammals have modified their digestive system morphology to allow prolonging gut retention time through the enlargement of the gut upstream or downstream of the stomach, thus generating the so-called foregut or hindgut fermenters, respectively (Stevens & Hume, 2004, Ley *et al.*, 2008).

In addition to host physiology and phylogeny, diet has been implied as another major drivers responsible for the modulation of the gut microbial community structure and functionalities. The mammalian gut microbial ecosystem is, indeed, able to respond to the host diet over both daily and evolutionary time scales (Ley *et al.*, 2008, Muegge *et al.*, 2011, McFall-Ngai *et al.*, 2013). In this context, several metagenomic-based analyses not only revealed strict associations between the composition of the intestinal microbial community and host diet, even among phylogenetically unrelated mammals, but they also highlighted diet contribution to shape the gut microbiome by selecting those metabolic activities that allow the degradation of the host diet-specific components. Thus, suggesting that the gut microbial consortium ensure complementary diet-dependent metabolic functions to the host (Ley *et al.*, 2008, Muegge *et al.*, 2011, Delsuc *et al.*, 2014). With respect to biodiversity, herbivore microbiota is generally characterized by the highest number of bacterial phyla, followed by omnivores and finally carnivores that possess the lowest bacterial richness level, a classification that is also reflected at the genus level (Ley *et al.*, 2008). At the same time, while the existence of herbivore- (members of the Firmicutes phylum) and carnivore-associated (representatives of the Fusobacteria phylum) bacterial lineages were discovered, suggesting functional adaptations of these microorganisms to host-specific diets, omnivores seemed not to possess any 'specialist' bacteria but rather a combination of the herbivorous and carnivorous bacterial groups, thus reflecting their promiscuous diet (Groussin *et al.*, 2017). In parallel to differences in terms of richness and taxonomic composition, diet is also responsible for shaping the genetic repertoire of a specific gut ecosystem in order to select those functional activities that are able to

breakdown the macromolecules typical of a specific diet. In this context, functional annotation of the genetic arsenal of herbivores and carnivores revealed that while the intestinal microbial community of carnivores is characterized by a wide range of genes involved in amino acid degradation pathways dedicated to protein digestion, the microbiome of herbivores is not only enriched in genes encoding for amino acids biosynthesis, useful to compensate for the reduced intake of protein-derived diet, but they also possess an arsenal of genetic sequences encoding enzymes involved in the breakdown of complex plant-derived glycans (Muegge *et al.*, 2011). Furthermore, members of the gut microbiota not only helped the host through food digestion but they are also involved in modifying xenobiotics from therapeutic drugs to diet-derived bioactive molecules. Indeed, especially herbivores, whose diet is based on plants that generally produce secondary compounds or dietary toxins, often rely on the capability of certain gut microorganisms to detoxify plant toxins by activating the production of specific enzymes (Dearing & Kohl, 2017, Suzuki, 2017). However, major dietary transitions are inevitably associated with other several physiological changes, thus making difficult to correlate a change in the gut microbiota of a specific mammal to its peculiar diet or phylogeny. From this perspective, several mammals harbour a gut microbial community that is opposite to the general pattern of convergence by diet. Despite their herbivorous diet, the Giant and Red Pandas' intestine not only is inhabited by microbial members similar to their carnivorous and omnivorous confamilials, but these microorganisms also substantially differ in functional gene complement from other herbivorous exponents (Ley *et al.*, 2008, Davenport *et al.*, 2017). In a similar way, despite the fact that their diet is exclusively based on animals such as fish and crustaceans, the baleen whale gut microbiota shares similarities in both taxonomy composition and genetic repertoire with the distantly related foregut-fermenting herbivores (Sanders *et al.*, 2015). Indeed, even if the baleen whale microbiome is enriched in genes catalysing glutamine and glutamate degradation, one of the diagnostic pathways that distinguishes a predominantly animal- from a plant-based diet, it also records a high abundance of genes encoding for carbohydrate degradation, especially involving activities related to the breakdown of chitin, a prominent polymer of the invertebrate exoskeletons in

which their diet is enriched (Sanders *et al.*, 2015). In this scenario, several studies have revealed that bats, which can be considered carnivorous, fructiferous or insectivorous depending on their dietary specialization, evolved conflicting patterns with respect to diet and gut microbiota composition (Phillips *et al.*, 2012, Carrillo-Araujo *et al.*, 2015). Therefore, in comparative analysis, both diet and host phylogeny should be taken into account since the modulation of the gut microbiota of mammals is the result of the combined influence of these two factors.

Another important force driving the mammalian gut microbiota modulation is represented by the anthropogenic influence. Selective pressure imposed by human intervention has been considered as a major player in several evolutionary events, encompassing the process of speciation leading to the creation of new mammalian clades generally obtained through domestication, relocation or novel ecosystem creation (Bull & Maron, 2016). Specifically, domestication, the process through which humans have deliberately modified feeding, breeding, environment and behaviour of animal species, not only has significantly altered the mammalian genes and associated phenotypic traits, but it has also shaped the intestinal microbial structure of domesticated mammals when compared to that of their close feral relatives (Metcalf *et al.*, 2017). Indeed, dietary alterations, reduced contact with natural environment and other animal species, antibiotic exposure and increased contact with humans are just some of the substantial alterations imposed on domesticated animals by anthropogenic forces that may have an impact on their gut microbial composition (Ushida *et al.*, 2016, Ferrario *et al.*, 2017, McKenzie *et al.*, 2017). In this context, several studies reporting a comparison of the gut microbiota of domesticated animals with that of their wild relatives, highlighted altered gut bacterial community (Scupham *et al.*, 2008, Muegge *et al.*, 2011, Nelson *et al.*, 2013).

D. Gut microbiota in companion animals

Starting from their initial domestication, initiated some 15,000 years ago, domesticated dogs and cats represent the first animals to have been subjected to profound anthropogenic influences, leading to the selection and subsequent global dissemination of countless canine and feline breeds. Across recent millennia, these two animals have gradually gained a special position within human society (Barker & Wolen, 2008). From predators living in the woods and open fields, dogs and cats now live within the domestic confinement of four walls, sharing all living space and sometimes a bed with their owner(s), thereby becoming the principal companion animals for humans (Dotson & Hyatt, 2008). Not by chance, the increasingly intimate relationship between these pets and their owners has promoted dogs and cats as integral part of a family unit (Cohen, 2002). As family members, concerns about canine and feline health and well-being have exponentially increased over time, opening a new veterinary research field aimed at defining strategies to promote health and longevity and, at the same time, prevent any diseases. In this context, since its involvement in influencing host health, the scientific community has dedicated particular interest to the study of the canine and feline intestinal ecosystem (Grzeskowiak *et al.*, 2015).

Gut microbiota composition in healthy dogs and cats

Culturing techniques coupled with NGS-based culture-independent approaches revealed different bacterial biodiversity and abundance along the canine and feline GIT (Ritchie *et al.*, 2008, Suchodolski *et al.*, 2008). Specifically, culture-based bacterial enumeration efforts have shown a gradual increment of the total bacterial load, ranging from 10^2 up to 10^{14} colony forming units (CFU) per gram of luminal content, starting from the stomach, passing through the small intestine to reach the large intestine where the highest bacterial abundance is recorded (Mentula *et al.*, 2005). In parallel, culture-independent molecular approaches highlighted similar variations in the bacterial biodiversity along the length of the GIT, with the distal intestinal tract of dogs and cats harbouring the greatest taxonomic richness when compared to the stomach and small intestine. Furthermore, it has been demonstrated that, while the foregut is generally colonized by both aerobic and facultative anaerobic microorganisms, the hindgut is almost exclusively colonized by anaerobic bacteria. Taken together, these observations strengthen the notion that the bacterial community along the GIT is not randomly assembled, but rather is the result of the various microenvironment and oxygen bioavailability as well as of the different physiological activities typical of each intestinal compartment that influence the gut community assembly (Suchodolski *et al.*, 2008, Suchodolski, 2016). Despite evidence of variations in terms of bacterial abundance and biodiversity along the GIT, most of the current knowledge about the gut microbiota of dogs and cats is limited to fecal sample-based analyses due to the practical difficulties and ethical constraints encountered in retrieving samples from each intestinal region. In addition, considering that non-standard experimental metagenomics protocols have been applied across different studies and that taxonomic inter-individual variations occur among animals of the same species due to several factors such as diet, age, gender, environment, genetics and diseases, the canine and feline intestinal community is far from being completely dissected. Despite these limitations, key bacterial players have been consistently found in fecal samples of healthy dogs and cats, allowing the definition of a core fecal

microbial community, i.e. intestinal bacterial members shared by dogs and cats throughout multiple studies (Minamoto *et al.*, 2012, Salonen *et al.*, 2012, Hand *et al.*, 2013). In this context, Bacteroidetes, Firmicutes and Fusobacteria represent the most abundant and prevalent bacterial phyla of the intestinal microbial consortium of both dogs and cats (Ritchie *et al.*, 2008, Handl *et al.*, 2011, Minamoto *et al.*, 2012, Tun *et al.*, 2012). Within the Bacteroidetes phylum, *Prevotella* and *Bacteroides* are two of the most abundant and prevalent bacterial genera characterizing the canine and feline intestinal ecosystem. On the other side, Bacilli, Clostridia and Erysipelotrichi represent the main bacterial classes among the Firmicutes phylum. In detail, the Clostridia class corresponds to the most abundant taxa and is dominated by three different clusters: *Clostridium* cluster IV (i.e. *Faecalibacterium prausnitzii* and *Ruminococcaceae* family), XI (i.e. *Peptostreptococcaceae* family) and XIVa (i.e. *Blautia* and *Lachnospiraceae* family) (Ritchie *et al.*, 2008, Ritchie *et al.*, 2010, Handl *et al.*, 2011). Besides Clostridia, the Bacilli class is represented by the *Enterococcus* and *Lactobacillus* genera in cats, while *Streptococcus* and *Lactobacillus* dominate the canine gut microbiota. The Erysipelotrichi, instead, mainly consists of the *Turicibacter*, *Catenibacterium* and *Coprobacillus* genera (Ritchie *et al.*, 2008, Ritchie *et al.*, 2010, Handl *et al.*, 2011). Despite the consolidate implication of the *Fusobacterium* genus, and especially the *Fusobacterium nucleatum* species, in human inflammatory bowel diseases with possible complications and linkages to colorectal cancer, this bacterial taxa belonging to the Fusobacteria phylum is a relevant microbial members of the canine and feline gut microbiota, even if with a lower average relative abundance in cats when compared to dogs (Ritchie *et al.*, 2008, Suchodolski *et al.*, 2008, Castellarin *et al.*, 2012, Gagniere *et al.*, 2016, Marmol *et al.*, 2017). However, this negative association between *Fusobacterium* spp. and intestinal cancers has not been observed in dogs and cats, while the common distribution of this bacterial genus in the intestinal tract of other carnivorous animals suggests that, different from what appears to be going on in humans, it is not harmful for animal hosts (Ley *et al.*, 2008, Vazquez-Baeza *et al.*, 2016). In this context, the most reliable hypothesis to explain the high abundance of the *Fusobacterium* genus in the gut microbiota of carnivores, including dogs and cats,

relies on the peculiar nutritional requirements and metabolic features of this taxon. Indeed, *Fusobacterium* spp. genomes possess a wide range of genes encoding for proteases, enzymes able to degrade proteins, main components of the canine and feline diet, in order to generate their elective growth substrates, i.e. peptides and/or amino acids (Doron *et al.*, 2014). Furthermore, some members of the *Fusobacterium* genus, including *Fusobacterium varium*, have been described not only as potent antagonists able to prevent pathogen colonization, but they are also able to activate protein fermentation with the subsequent production of butyrate, one of the main SCFA produced by fermentative bacteria in the intestine, that ultimately exerts anti-inflammatory effects and serves as nutritional sustenance for enterocytes (Potrykus *et al.*, 2008).

Although with reduced relative abundance respect to the co-dominant Bacteroidetes, Firmicutes and Fusobacteria phyla, the Actinobacteria and Proteobacteria taxa are other two prevalent exponents of the canine and feline intestinal microbial population (Ritchie *et al.*, 2008, Suchodolski *et al.*, 2008, Tun *et al.*, 2012, Deng & Swanson, 2015). In particular, while members of the Actinobacteria are more abundant in the feline GIT, Proteobacteria representatives show higher relative abundance in the canine intestine (Handl *et al.*, 2011). Specifically, within the Actinobacteria phylum, the *Collinsella* and *Slakia* genera dominate canine fecal samples, while the feline gut microbiota is characterized by high abundance of *Eggerthella* and *Olsenella (Handl et al., 2011)*. On the other side, the Proteobacteria phylum is mainly represented by *Anaerobiospirillum*, *Escherichia/Shigella*, *Succinovibrio* and *Sutterella* in both canine and feline intestinal consortia (Handl *et al.*, 2011).

Although a core fecal microbiota has been described, several factors, including diet (Kim *et al.*, 2017), age (Masuoka *et al.*, 2017), anthropogenic influences or metabolic disorders (Kil & Swanson, 2011, Suchodolski, 2016) may modulate the taxonomic composition of the canine and feline gut microbiota or may even led to aberrant alterations in case of intestinal bowel diseases or cancer (Honneffer *et al.*, 2014, Xu *et al.*, 2016).

The impact of dietary interventions on the gut microbiota of dogs and cats

Originally classified as carnivores, in the course of the domestication process, dogs have undergone radical changes in their dietary habits. Shifting from eating prey to commercial pet foods formulated to guarantee a balanced nutritional intake, including a high concentration of fibres and carbohydrates required for the production of extruded dry food, the domesticated dog is currently considered as an omnivorous mammal (Swanson *et al.*, 2011). In contrast, due to the evolutionary adaptations to a strictly carnivorous diet, rich in protein and fat, that have shaped the feline anatomy by selecting a short colon and the lack of a functional cecum, the domestic cat is still effectively classified as a carnivorous animal (Rochus *et al.*, 2014). However, despite the peculiar feline intestinal anatomy, several studies reported the capability of the gut microbial community of cats to ferment various dietary plant-derived fibres in which pet commercial foods are enriched (Hooda *et al.*, 2013). Although vegetable origin components were initially incorporated in the formulation of commercial pet foods exclusively for production purposes, in recent years pet food industries have dedicated special efforts to carefully identify the best blend of dietary fibres to be included in the final product in order to obtain functional foods aimed at improving pet health (de Godoy *et al.*, 2013). Indeed, dietary fibres have been reported to influence several physiological aspects, including modulation of the intestinal peristalsis affecting laxation and stool quality as well as contribution to weight loss and subsequent reduction of the incidence of obesity and diabetes in pet population (de Godoy *et al.*, 2013). Specifically, as a consequence of the health-promoting effects observed in humans, special attention has been dedicated to a small group of mostly plant-derived fibres and carbohydrates, the so-called prebiotics, non-digestible carbohydrates ranging from the disaccharides lactulose to oligo- or poly-saccharides, including inulin or fructo-oligosacchrides (FOS). To be effective, prebiotics have to escape hydrolysis by the upper GIT host enzymes in order to reach the hindgut where they can exert their beneficial effects to the host by stimulating the proliferation and/or metabolic activities of

specific beneficial bacterial species (Pinna & Biagi, 2016). By withstanding the upper intestinal digestion and absorption and then reaching the distal part of the intestine, prebiotics are selectively digested by those microorganisms whose genomic assembly contains genes encoding for extra- and/or intra-cellular GHs and transport system, involved in the degradation and uptake of these complex carbohydrates, respectively (Vieira *et al.*, 2013, Pinna & Biagi, 2016). Beyond acting as carbon sources for the selective proliferation of beneficial bacterial species, including *Bifidobacterium* spp., *Lactobacillus* spp. or butyrate-producing bacteria such as *Faecalibacterium prausnitzii* or *Eubacterium hallii* in the canine and feline intestine (Barry *et al.*, 2010, Middelbos *et al.*, 2010, Grzeskowiak *et al.*, 2015, Segarra *et al.*, 2016), the prebiotic fermentation in the distal intestinal tract favours the accumulation of SCFAs, mainly represented by butyrate, acetate and propionate, which are able to improve host health by acting as energy substrates for enterocytes and peripheral tissue, modulating the immune system, protecting from the proliferation of pH-sensitive pathogenic microorganisms through the acidification of the luminal environment, regulating bowel activities and favouring mineral absorption (Slavin, 2013, Vieira *et al.*, 2013, Pinna & Biagi, 2016). However, despite the well-accepted health-promoting effects of prebiotics and the continuous and extensive dedication of pet food industries to the development of new recipes to guarantee a balanced pet diet, in recent years an opposing trend has been observed with a growing preference among pet owners to return to a more natural and home-made diet, feeding dogs and cats with the so-called Raw Meat-Based Diet (RMBD) rather than the conventional commercial dry or wet food. The RMBD formulation principally includes raw meat, fat, internal organs, cartilage, fleshy bones and skeletal muscles from farm animals, horses, game and fish, but it can also contain eggs, dairy products or vegetables even if at a largely reduced concentration when compared to the extruded diet (Fredriksson-Ahomaa *et al.*, 2017). Investigations aimed at dissecting the advantages and disadvantages of feeding pets with a natural diet have highlighted important health benefits for the pet consumer, encompassing fresher breath and reduced incidence of dental diseases, alleviation of arthritis and allergies (Freeman *et al.*, 2013). On the other side, the administration of RMBD was

discouraged since it does not ensure a balanced nutritional intake and it is directly related to high risk of exposure to zoonotic pathogens for both pets and owners (Schlesinger & Joffe, 2011, Kim *et al.*, 2017). Furthermore, differences in the macronutrient composition between RMBD formulation and commercial pet food play a relevant role in the modulation of both taxonomic composition and metabolic functionalities of the gut microbiota of dogs and cats (Fredriksson-Ahomaa *et al.*, 2017, Kim *et al.*, 2017). In this context, RMB diet promotes the proliferation of two protein-degrader bacterial taxa, i.e. the *Clostridiaceae* family and *Fusobacterium* genus. Indeed, a positive correlation was observed between *Clostridiaceae* and protein dietary content as well as protein digestibility, thus suggesting the involvement of this taxon in protein digestion (Bermingham *et al.*, 2017). In support of this supposition, a significantly higher relative abundance of certain *Clostridium* species, including *Clostridium hiranonis*, *Clostridium perfringens*, *Clostridium ramosum* and *Clostridium rectum* was observed in healthy dogs fed on a RMB diet when compared to dogs fed on commercial pet foods (Kim *et al.*, 2017). Furthermore, a recent functional characterization of the gut microbiota of carnivores revealed that the genetic repertoire of *Clostridaceae*, especially *Clostridium perfringens*, is provided with a butyrate-kinase encoding gene that, belonging to the main butyrate-synthesis pathway, is responsible for the production of butyrate from proteins (Vital *et al.*, 2015). In a similar way, as already mentioned, members of the *Fusobacterium* genus are butyrate-producing bacteria via protein fermentation (Potrykus *et al.*, 2008). Apart from the marked increase of proteolytic bacteria, an RMB diet also favours a significant reduction in the relative abundance of saccharolytic taxa, i.e. members of the *Ruminococcaceae* family as well as *Faecalibacterium* and *Prevotella* genera, generally associated with a diet enriched in fibre and carbohydrates for their ability to breakdown a large amount of different dietary glycans (David *et al.*, 2014, Herstad *et al.*, 2017).

Effect of human-pet interplay on the gut microbiota of companion animals

Domesticated dogs and cats, as direct descendants of the Eurasian grey wolf and wild cat respectively, are among the first animals to have undergone extensive and profound anthropogenic influences (Savolainen *et al.*, 2002). The selective pressure imposed by humans has not only contributed to altered phenotypic and genotypic traits leading to the selection and diffusion of multiple canine and feline breeds, but it has also significantly impacted the intestinal microbial composition of domesticated animals if compared to that inhabiting their close, yet wild relatives (Ferrario *et al.*, 2017, McKenzie *et al.*, 2017, Wu *et al.*, 2017). In this context, a metagenomics-based study revealed differences in the taxonomic composition of the gut microbiota of wolves when compared to that of their domesticated counterparts. While the *Streptococcus* genus and members of the *Verrucomicrobia* phylum were only detected in the canine feces, representatives of the *Cyanobacteria* taxon were exclusively found in wolves, thus suggesting that human interference has shaped the canine gut composition (Wu *et al.*, 2017). Furthermore, the radical changes in canine lifestyle and dietary habits due to the domestication process, have favoured the enrichment of certain bacterial taxa generally associated to a carbohydrate-rich diet, encompassing *Carnobacillus*, *Desulfuromusa, Faecalibacterium*, *Lactobacillus* and *Ruminococcaceae* and the subsequent increment of genes involved in carbohydrate metabolism pathways in the canine intestinal consortium respect to that of wolves (Lyu *et al.*, 2018). Although there is currently no evidence of the modulation of both composition and metabolic repertoire of the bacterial intestinal population of cats by human influence, one may assume that alterations observed in dogs may have occurred also in feline gut microbiota.

E. The *Bifidobacterium* genus: a commensal taxon of the mammalian gut microbiota

General features and ecology of the *Bifidobacterium* genus

Members of the *Bifidobacteriaceae* family, bifidobacteria are Gram-positive, non-motile, non-spore-forming, anaerobic and saccharolytic microorganisms characterized by a high G+C DNA content (Turroni *et al.*, 2009). Starting from the description of the first bifidobacterial species, isolated from the fecal sample of a breast-fed infant by Tissier in 1899 (Tissier, 1900), the number of species belonging to the *Bifidobacterium* genus is constantly updated, with several of the currently recognized species that have only recently been isolated from different species of monkeys, i.e. *Bifidobacterium aerophilum*, *Bifidobacterium avesanii*, *Bifidobacterium ramosum, Bifidobacterium myosotis, Bifidobacterium tissieri, Bifidobacterium hapali* and *Bifidobacterium vansinderenii* (Ventura *et al.*, 2007, Michelini *et al.*, 2016, Michelini *et al.*, 2016, Duranti *et al.*, 2017). In this context, with 55 currently recognized taxa, involving 46 species and nine subspecies, the *Bifidobacterium* genus forms the deepest branching lineage within the Actinobacteria phylum (Ventura *et al.*, 2007, Lugli *et al.*, 2017). A phylogenomic analysis based on the concatenation of 314 protein sequences representing the *Bifidobacteriaceae*-specific clusters of orthologous genes, generated a robust phylogenetic super-tree showing the division of the *Bifidobacterium* genus into seven different phylogenetic clusters, including *Bifidobacterium adolescentis*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium boum*, *Bifidobacterium longum*, *Bifidobacterium pseudolongum* and *Bifidobacterium pullorum* (Lugli *et al.*, 2017) (Figure 2).

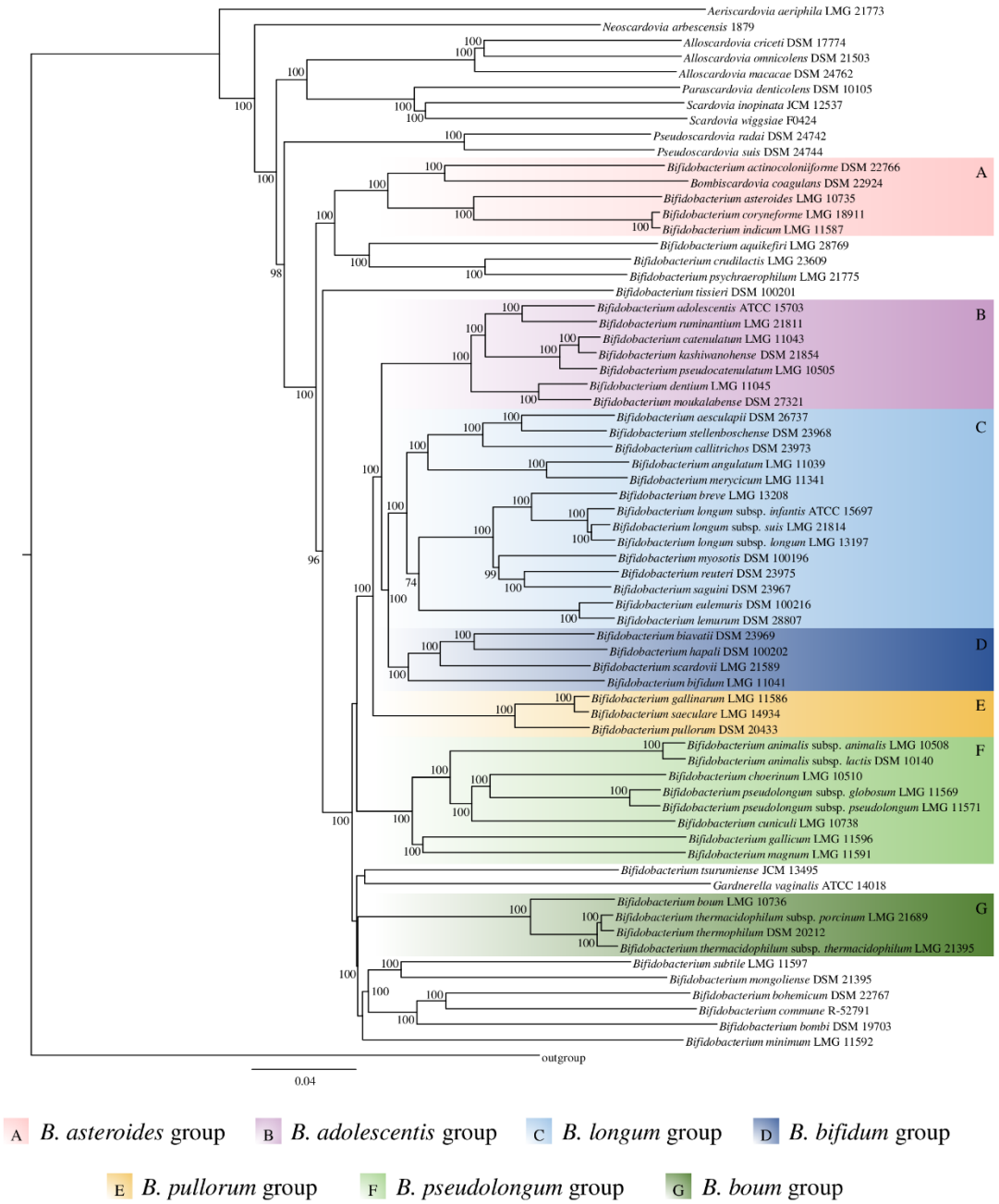

Figure 2. Supertree of the *Bifidobacteriaceae* family based on the concatenation of the amino acids sequences from 314 core genes. The seven bifidobacterial groups are highlighted with different colours.

Specifically, the *B. asteroides* phylogenetic group was pointed out as the evolutionary ancestor of the *Bifidobacterium* genus, since it positioned itself as the group closest to the root of the generated family-based super-tree (Milani *et al.*, 2016, Lugli *et al.*, 2017). However, the recent wide diffusion of 16S rRNA gene and bifidobacterial ITS microbial profiling for the investigation of the taxonomic composition of the mammalian gut microbiota allowed the identification of putative novel members of the *Bifidobacterium* genus, thus making a further expansion and refinement of this phylogenetic classification predictable (Zoetendal *et al.*, 2008, Turroni *et al.*, 2012, Milani *et al.*, 2017).

Despite the multiple ecological niches from which bifidobacterial species have been isolated, i.e. human blood (Hoyles *et al.*, 2002), oral cavity (Okamoto *et al.*, 2008), sewage (Biavati *et al.*, 1982), raw or fermented milk (Delcenserie *et al.*, 2007, Watanabe *et al.*, 2009) and intestine of insects and birds (Trovatelli *et al.*, 1974, Praet *et al.*, 2015), the vast majority of bifidobacterial species are associated with the GIT of humans and other mammals (Killer *et al.*, 2011, Modesto *et al.*, 2015, Michelini *et al.*, 2016). Indeed, members of the *Bifidobacterium* genus are classified as abundant components of the intestinal microbial community of multiple animals that provide parental care to their offspring, encompassing both warm-blooded mammals and social insects (Bunesova *et al.*, 2014, Milani *et al.*, 2017). Despite their broad ecological distribution, for a long time, it has been inferred that the ability of members of the *Bifidobacterium* genus to colonize different ecological niches is species-dependent. In this context, while some bifidobacterial taxa seemed to enjoy a cosmopolitan lifestyle, such as *B. adolescentis*, *B. bifidum*, *B. longum* and *B. pseudolongum* (Turroni *et al.*, 2011, Milani *et al.*, 2017), other species appeared to have evolved the ability to adopt to a specific animal gut ecosystem, such as *Bifidobacterium angulatum*, *Bifidobacterium cuniculi* or *Bifidobacterium gallinarum* in cows, rabbits and chickens, respectively (Turroni *et al.*, 2011). Similarly, a group of six bifidobacterial species was traditionally considered to be highly specialized in the colonization of the intestine of social insects, encompassing *B. asteroides*, *Bifidobacterium actinocoloniiforme*, *Bifidobacterium bohemicum*, *Bifidobacterium bombi*, *Bifidobacterium coryneforme* and *Bifidobacterium indicum* (Bottacini et al., 2012). However, the application of

metagenomic techniques, especially the bifidobacterial ITS microbial profiling, to a large spectrum of mammalian species have completely revolutionized the ecological classification of the bifidobacterial species, revealing that even with a reduced relative abundance when compared to the cosmopolitan bifidobacteria, most bifidobacterial taxa previously identified as niche-specialized are able to colonize a wide number of mammalian host, thus refuting the supposed strict host-related specialization behaviour but rather suggesting a strain-specific adaptation (Milani *et al.*, 2017). This hypothesis was further supported by the observation that also the intestine of carnivorous mammals, whose diet is poor in fermentable carbohydrates that represent the preferred substrates for bifidobacterial growth, is inhabited by bifidobacterial species (Milani *et al.*, 2017). Specifically, their adaptation to different ecological environment strictly relies on the peculiar genetic assembly of each bifidobacterial taxa, thus suggesting that the metabolic strategies employed by these commensal microorganisms play a crucial role in underpinning their ecological adaptation to, and fitness in the mammalian GIT (Milani *et al.*, 2015).

In the last 20 years, among the multitude of microorganisms that inhabit the mammalian gut microbiota, the scientific community has dedicated a particular interest to bifidobacteria due to their ability to exert a multitude of health-promoting effects to the host. Consequently, several members of the *Bifidobacterium* genus are currently exploited as valid probiotic bacteria. Specifically, bifidobacterial species can promote beneficial effects by degrading complex diet-derived otherwise non-digestible carbohydrates (glucans, xylans, cellulose, fructans or pectins) as well as host-derived glycans such as Human Milk Oligosaccharides (HMOs) and mucins (Pokusaeva *et al.*, 2011, O'Callaghan & van Sinderen, 2016). In addition to this nutrient assimilation ability, bifidobacteria are also able to exert many other beneficial effects to the host at both local and systemic levels, preventing invasion and subsequent colonization by pathogen microorganisms, modulating gut homeostasis, educating and stimulating the host immune system by soliciting adaptive and/or innate immune responses (Hidalgo-Cantabrana *et al.*, 2017, Ruiz *et al.*, 2017).

Bifidobacterium genus adaptation to and interaction with the GIT environment

In order to colonize the human and animal gut, bifidobacteria have to counter the hostile and adverse conditions typical of the upper parts of the GIT. Exposure to oxygen or oxygen-derived free radicals, organic and bile acids or osmotic stress are just some of the factors that can negatively impact on the bifidobacterial cell viability and functionality (Bottacini *et al.*, 2014). To counteract these stressful conditions, bifidobacteria rely on a tight control of a repertoire of genomic mechanisms, including molecular chaperons, bile salt hydrolases, bile efflux transporters as well as two component systems and ATPases, to rapidly react to several complex environmental challenges (Turroni *et al.*, 2010, Alvarez-Martin *et al.*, 2012, Ruiz *et al.*, 2012, Ruiz *et al.*, 2012). In this context, comparative genome analysis revealed that all members of the *Bifidobacterium* genus adopt a universal system in response to osmotic, oxidative and acid stress, since the stress response regulation is driven by an interactive network of regulators whose genetic sequences are highly conserved across the genome of currently sequenced bifidobacterial species (Zomer *et al.*, 2009, Bottacini *et al.*, 2014). Specifically, bile toxicity is countered by the activation of efflux pumps involved in the extrusion of bile salts and acids from the cytoplasm to the extracellular environment, ensuring the survival of bifidobacteria during the transit through the small intestine (Price *et al.*, 2006, Gueimonde *et al.*, 2009, Ruiz *et al.*, 2012). Furthermore, in order to withstand the competitive and hostile GIT environment, some bifidobacterial species have evolved the ability to protect themselves against host proteases through an environmental sensing two component system that, when activated, is able to induce the production of serine protein inhibitors, typically known as serpins, acting as eukaryotic-type serine protease inhibitors (Potempa *et al.*, 1994, Turroni *et al.*, 2010, Alvarez-Martin *et al.*, 2012). Once the upper part of the GIT has been passed, bifidobacteria finally reach their elective ecological niche, i.e. the large intestine, where they activate multiple strategies to interact with the host and ensuring their fitness in the intestinal environment. Although the molecular mechanisms through which bifidobacteria dialogue with the host are still under scrutiny, the genome sequencing of known taxa

of this bacterial genus helped to identify which genes may actually be involved in the microbe-host interactions (Fanning *et al.*, 2012, Turroni *et al.*, 2013). Specifically, a set of extracellular structures, excreted enzymes and bioactive metabolites have been identified as pioneering bifidobacteria-produced macromolecules responsible for the interaction with the host. Among others, hair-like proteinaceous appendages protruding from the extracellular cell surface, known as pili or fimbriae, are directly involved in the aggregation with other intestinal microorganisms or in biofilm formation as well as in the adhesion to the intestinal epithelium, thus playing a crucial role for successful colonization of a specific environment (Schell *et al.*, 2002, Foroni *et al.*, 2011, Turroni *et al.*, 2013, Milani *et al.*, 2017). In bifidobacteria, two different types of pili have been observed, i.e. sortase-dependent pili and Tight Adherence Pili (Tad pili) which differ for biogenesis system and genetic loci sequences (O'Connell Motherway *et al.*, 2011, Milani *et al.*, 2017). Another important extracellular structure is represented by the so-called exopolysaccharides (EPS), glycan layers that form an external envelope in a wide spectrum of bacteria (Perez *et al.*, 1998, Ventura *et al.*, 2007, Ruas *et al.*, 2009). EPS macromolecules are not only able to promote host colonization and persistence, but they also confer to EPS-producing bifidobacteria protection against adverse conditions such as drastic changes in the pH environment or presence of bile acids (Ruas-Madiedo *et al.*, 2009, Fanning *et al.*, 2012). Furthermore, both pili and EPS are involved in establishing a bifidobacteria-host immune system dialogue contributing to prime and educate the host immune system, especially in the newborns where the bifidobacterial population reach the highest abundance during the life-span of a mammalian host, as well as to induce the maturation and proliferation of the epithelial colonic cells, thus incrementing host mucosal homeostasis (Hidalgo-Cantabrana *et al.*, 2017).

Insight into the saccharolytic features of the *Bifidobacterium* genus

The advent of NGS techniques have not only allowed large-scale metagenomic studies, but it has also offered the possibility of sequencing single genomes thus making comparative genome analyses extremely informative in order to better understand the processes that have influenced bacterial speciation and evolution, as well as their adaptation to a specific ecological environment. In this context, a comparative genome analysis involving 47 *Bifidobacterium* (sub)species allowed the identification of 18,181 and 551 *Bifidobacterium*-specific Clusters of Orthologous Genes (BifCOGs) representing the bifidobacterial pan- and core-genome of the *Bifidobacterium* genus, respectively (Milani *et al.*, 2014, Milani *et al.*, 2015). Specifically, functional annotation of the core BifCOGs revealed that, while a major part of the bifidobacterial core sequences correspond to housekeeping genes, several other core genes are involved in the adaptation and interaction with a specific environment, encompassing genes encoding for carbohydrate breakdown (Milani *et al.*, 2015). At the same time, the pan-genome analysis not only revealed that most of the pan BifCOGs is represented by COG families corresponding to carbohydrate metabolism functions, but it also allowed the identification of the so-called Truly Unique Genes (TUGs), i.e. genes that have been exclusively found in just one of the examined bifidobacterial species, whose functional annotation showed their involvement in carbohydrate metabolism, encompassing GHs and carriers for glycan uptake (Milani *et al.*, 2015). To support these *in silico* observations, a large number of *in vitro*-based published studies reported the ability of bifidobacteria to degrade a wide variety of complex diet-derived carbohydrates otherwise non-digestible by the host, including glucans, pectins, cellulose, starch, arabinogalactans, fructans and xylans, as well as host-derived glycans such as Human Milk Oligosaccharides (HMOs), typical complex carbohydrates of the human milk, and mucins, glycoproteins that cover the mammalian gut epithelium (Turroni *et al.*, 2010, Pokusaeva *et al.*, 2011, Turroni *et al.*, 2011, Egan *et al.*, 2014, O'Callaghan & van Sinderen, 2016). The genetic arsenal required for the breakdown of these complex sugars are generally arranged in specific clusters

containing extracellular and/or intracellular GH-encoding genes coupled with genetic sequences that encode for proteins involved in carbohydrate transport (Pokusaeva *et al.*, 2011, Bottacini *et al.*, 2017). Once degraded, internalized and possibly further modified, carbohydrates converge to the final metabolic route, known as "bifid shunt", at the end of which not only energy is produced in the form of ATP and glucose for the sustenance of the bacterial cell itself, but also SCFAs and other organic acids are released, mainly encompassing lactate and acetate (De Vuyst *et al.*, 2014). The latter have important health-promoting repercussion on the host, since SCFAs are generally involved in increasing mineral absorption, providing nutritional support to enterocytes, regulating bowel functions and reducing the luminal pH, thus limiting the prevarication of pH-sensitive pathogenic microorganisms (den Besten *et al.*, 2013). Furthermore, SCFAs produced by bifidobacteria can be exploited by other members of the gut microbiota to produce different SCFAs than acetate, such as butyrate that is involved in driving the immune system through an anti-inflammatory response (den Besten *et al.*, 2013). In addition to enhancing SCFA production in the intestine, by exploiting their saccharolytic potential, bifidobacteria also play an ecological role in shaping the gut microbiota composition by promoting cross-feeding events (Milani *et al.*, 2016). Indeed, their capability to degrade those polysaccharides that reach the hindgut intact through the production of extracellular GHs allows the release in the extracellular environment of simple glycans, i.e. mono- or oligo-saccharides that become available to other bifidobacterial species or other gut microbial members that lack, in their genetic repertoire, the specialized enzyme to degrade complex carbohydrates (den Besten *et al.*, 2013, Milani *et al.*, 2016). In this context, bifidobacteria were depicted as microorganisms with a social intelligence for their mutualistic/commensal abilities to target carbohydrates aimed at regulating the dynamics of gut microbiota interactions (Milani *et al.*, 2015, Milani *et al.*, 2016, Turroni *et al.*, 2016).

Chapter 2

Materials and methods

Ethical statement

All studies were performed in compliance with the rules, regulations and recommendations of the Ethical Committee of the University of Parma. Experimental procedure and protocols were approved by the 'Comitato di Etica Università degli studi di Parma', Italy. Furthermore, all animal procedures were carried out following national guidelines (decreto legislative 26/2014), while a written consent to participate were obtained from human participants before their enrolment in the studies.

Sample collection and DNA extraction

Stool samples from dogs and cats were collected through a collaboration with several Italian dog and cat breeders located in the North and centre of Italy, while stool samples from other mammalian species were retrieved through a collaboration with several Italian zoological parks, farms, livestocks as well as with the Natural Park of Abruzzo (Italy) for wild animals. Furthermore, in the case of aquatic mammals, samples were collected during a routine veterinary examination through rectal swabs in order to avoid contaminations. In general, to be included in the study, animals had to be healthy without having been exposed to any treatment with prebiotics, probiotics or drugs, such as antibiotics, during the six months prior to sample collection. In addition, for each sample, mammalian species or even breed for dogs and cats were noted and, where possible also age, gender and weight were annotated. In all cases, stool samples were collected immediately after defecation in a suitable sterile container, kept on ice and shipped to the laboratory under frozen conditions. Once in the laboratory, samples were divided into two different aliquots. A first aliquot was transferred into a new fecal container with RNA later and conserved at -20°C for any future RNA extractions. The second aliquot, on the other hand, was preserved at -20°C without adding any solution, until they were processed. DNA extraction from every single fecal sample was performed using the QIAmp DNA Stool Minikit following the manufacturer's instructions (Qiagen, Germany).

16S rRNA gene/bifidobacterial ITS sequencing

Partial 16S rRNA gene sequences were amplified from extracted DNA exploiting the primer pair Probio_Uni (5'-CCTACGGGRSGCAGCAG-3') and Probio_Rev (5'-ATTACCGCGGCTGCT-3'), targeting the V3 region of the 16S rRNA gene sequence of the ribosomal locus (Milani *et al.*, 2013). Conversely, Partial ITS sequences were PCR-amplified from extracted DNA using the primer pair Probio-bif_Uni (5'-CTKTTGGGYYCCCKGRYYG-3') and Probio-bif_Rev (5'-CGCGTCCACTMTCCAGTTCTC-3'), which targets the spacer region between the 16S rRNA gene and 23S rRNA gene within the rRNA locus (Milani *et al.*, 2014). In both cases, Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons and to the targeted ITS amplicons, which were further processed following the Illumina instructions regarding the 16S metagenomic sequencing library preparation protocol (part number 15044223 rev. B; Illumina). Amplifications were performed using a Verity thermocycler (Applied Biosystem, USA). The integrity of the obtained PCR amplicons was visualized by electrophoresis by using a 2200 Tape Station instrument (Agilent Technologies, USA). Subsequently, DNA products generated through the PCR-mediated amplification of a partial sequence of the 16S rRNA genes were purified by means of a magnetic purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) to remove primer dimers. At this point, DNA concentration of the amplified sequence library was determined by a fluorometric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a final concentration of 4 nM and 5 µl of each diluted DNA amplicon sample were mixed to generate the final pooled library. 16S rRNA gene sequencing was performed using an Illumina MiSeq sequencer with MiSeq reagent kit V3. Each step of the library preparation were carried out by employing HiPure molecular biology-grade water (GE Healthcare, USA). In addition, a negative control was sequenced in order to verify that any contamination did not occur during the amplification and sequencing steps. Specifically, the

negative control was prepared as a normal samples by substituting DNA with HiPure molecular biology grade-water.

16S rRNA gene/bifidobacterial ITS microbial profiling analysis

Following sequencing, the generated .fastq files were processed using QIIME2 software with the SciKit-learn classifier (Bokulich *et al.*, 2018). Specifically, paired-end reads were merged and then subjected to quality control in order to retain only those sequences with a length between 140 and 400 bp and mean sequence quality score of >20, while sequences with homopolymers of >7 bp and mismatched primers were omitted. 16S rRNA gene and bifidobacterial ITS Operational Taxonomic Units (OTUs) were defined at 100% sequence homology using DADA2 (Callahan *et al.*, 2016), thus generating the so-called Exact Sequence Variants (ESVs). ESVs not including at least two sequences of the same sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 (Caporaso *et al.*, 2010, Bokulich *et al.*, 2018) for both 16S rRNA gene and ITS sequences, while a reference data set from the SILVA database (Quast *et al.*, 2013) and a custom bifidobacterial ITS database (Milani *et al.*, 2014) were employed to analyse 16S rRNA gene sequences and bifidobacterial ITS sequences, respectively. Depending on the study, biodiversity was calculated using different parameters. Specifically, alpha-diversity was assessed with Chao1 richness, Shannon diversities or Phylogenetic Diversity (PD) indexes as well as with the observed OTU index. Similarity between samples, i.e. beta-diversity, was calculated by weighted UniFrac or Bray-Curtis and represented by Principal Coordinate Analyses (PCoA) using QIIME2 (Caporaso *et al.*, 2010, Bokulich *et al.*, 2018), while Good's coverage was calculated as $G=1-n/N$, where n is the number of singleton phylotypes and N is the total number of sequences in the sample.

Shotgun metagenomics and analysis of metagenomic datasets

The extracted DNA was prepared according to the Illumina Nextera XT protocol. Specifically, DNA samples were enzymatically fragmented, barcoded and purified involving magnetic beads.

Subsequently, samples were quantified through the fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 Tape Station Instrument (Agilent Technologies, USA) and normalized to 4 nM. A single-end sequencing was performed using and Illumina NextSeq 500 sequencer with NextSeq High Output v2 kit (Illumina Inc., San Diego, USA).

After sequencing, the obtained .fastq files were filtered for reads with a quality <25, for reads > 80 bp and for sequences of the mammalian host DNA through mapping with BWA software (Li & Durbin, 2009) against a custom database. Moreover, bases were removed from the end of the reads unless the average quality score was > 25, in a window of 5 bp. Only paired data were used for the subsequent analysis with the METAnnotatorX using default settings (Milani *et al.*, 2018). Furthermore, RapSearch2 software (Zhao *et al.*, 2012) and the CAZy database (Lombard *et al.*, 2014) were used to investigate the glycobiome profiles, while the MetaCyc database was exploited to predict the bacterial metabolic pathways (Caspi *et al.*, 2008).

RNA extraction from fecal samples

RNAlater-preserved fecal samples were vortexed and homogenized after thawing for 15 minutes. Approximately 0.4 g of stool slurry were mixed with 1 ml of QIAzol Lysis Reagent (Qiagen, Germany) in a sterile tube containing glass beads (Merck, Germany). The lysis of the bacterial cells was achieved by alternating for three times 2 minutes of stirring the mix on a Precellys 24 Homogenizer (Bertin Instruments, France) with 2 minutes of static cooling in ice. The lysed cells were centrifuged at 12,000 rpm for 15 minutes and the upper phase was recovered. Subsequently, the extracted RNA was purified using the RNAeasy Mini Kit (Qiagen, Germany) following the manufacturer's instruction. RNA concentration and purity were assessed by a Picodrop microliter spectrophotometer (Picodrop, UK).

RNA sequencing and analysis

2.5 µg of total RNA was processed to remove ribosomal RNA through the Ribo-Zero Magnetic Kit (Illumina, San Diego, USA) followed by purification of the rRNA-depleted sample by ethanol precipitation. Subsequently, RNA was prepared following the manufacturer's protocol (Illumina). The quality of the rRNA-depleted RNA samples was checked by a 2200 Tape Station (Agilent Technologies, USA). Then, a whole transcriptome library was constructed using the TruSeq Stranded RNA LT Kit (Illumina) and loaded into a NextSeq High Output v2 Kit as indicated by the technical support guidelines. After sequencing, adapter sequences were depleted from the obtained reads that were quality filtered with overall quality, quality window and length filters. Sequences corresponding to the mammalian host genomes were removed by mapping with BWA software (Li & Durbin, 2009) against a custom database for host genomes. Reads that passed the filtering steps were analysed with METAnnotatorX tool (Milani *et al.*, 2018). Counts of reads that correspond to ORFs were performed using HTSeq and analysis of the RPKM values was performed using the formula RPKM=numReads/(geneLenght/1000*totalNumReads/1,000,000).

Statistical analyses

All statistical analyses, i.e. Student's T-test, ANOVA, PERMANOVA and Kendall tau rank co-variance were performed with the SPSS software IBM SPSS Statistics for Windows, version 22.0; IBM Corp., Armonk, NY, USA). ANOSIM (analysis of similarity) statistical analysis was calculated by means of QIIME2, while ANCOM (analysis of the composition of microbiomes) statistical analysis and False Discovery Rate (FDR) were calculated as previously described (Glickman *et al.*, 2014, Gloor *et al.*, 2017). Force-driven network representations were obtained with the Gephi software (Bastian, 2009). Network clusters were predicted through the modularity statistical function of the Gephi software. The software MeV v 4.9.0 was used to construct hierarchical clustering based on the Pearson correlation distance matrix.

Recovery of bifidobacteria on selective media

One gram of a given fecal sample was mixed with nine ml of phosphate-buffered saline (PBS) solution, pH 6.5. Serial dilution and subsequent platings were assessed sing de Man-Rogosa-Sharpe (MRS) agar (Scharlau Chemie, Spain) supplemented with 0.05% (w/v) L-cysteine hydrochloride (Merck, Germany) and 50 µl/ml mupirocin (Delchimica, Italy). Agar plates were incubated in an anaerobic chamber (2.99 % H_2, 17.01 % CO_2 and 80% N_2) (Concept 400, Ruskinn) at 37°C for 48h. Twenty colonies were taken to adequately represent the bacterial biodiversity grown in the selective medium for each samples, resuspended in MRS broth and incubated in an anaerobic atmosphere at 37°C for 24h. Subsequently, DNA was extracted from each bacterial culture using a rapid mechanical lysis, as previously described (Turroni *et al.*, 2009) and identified at a (sub)species level through Internal Transcribed Spacer (ITS) amplification and DNA sequencing.

Chapter 3

Outline of the thesis

The focus of this PhD thesis is to investigate the taxonomic composition and functional activities of the intestinal microbial community of various mammalian species through the application of a multi-omics approach involving both metagenomics, encompassing 16S rRNA microbial profiling, bifidobacterial ITS microbial profiling and shotgun metagenomics, and metatranscriptomics in order to shed light on those factors affecting the gut microbiota of mammalian hosts. Indeed, thanks to the advances in the molecular field, evidences that the intestinal microbial community plays a crucial role in determining the health status of a host have been emerged. In this context, in recent decades more and more attention has been directed to the characterization of the human gut microbiota in order to unveil the microbe-microbe and microbe-host interactions that influence human health, however limiting the knowledge about the impact that the intestinal microbial consortium may have on non-human hosts.

Chapter 4 investigates the taxonomic composition of the gut microbiota of one of the principal companion animals for humans, i.e. the domesticated dog. Besides the description of the canine 'core gut microbiota', it aims at illustrating how some factors such as diet, anthropogenic influences and age impact on the canine core gut microbiota composition and metabolic functions, in addition to a focus on the bifidobacterial population that inhabit the canine intestine.

Chapter 5 describes the differences that occur between the canine and feline gut microbiota composition at the genus level. Furthermore, it provides a species-specific investigation, based on the bifidobacterial ITS microbial profiling, aimed at dissecting the composition of the bifidobacterial population inhabiting the intestine of dogs and cats and predicting how the bifidobacterial species of this communities interact with each other.

Chapter 6 discusses the role that anthropogenic forces have exerted in the modulation of both taxonomic composition and functional activities of the intestinal microbial community of mammals at a genus level as well as their impact in modulating the *Bifidobacterium* genus-associated population, involving several dyads of mammals formed by domesticated animals and their close, yet wild relatives.

Chapter 7 illustrates how diet and host digestive system physiology impact on the composition and functionality of the intestinal microbial consortium of a wide range of mammalian species covering most of the mammalian branch of the tree of life. This purpose has been achieved not only by using metagenomics techniques, but also involving metatranscriptomics in order to overcome the simplistic prediction of the metabolic pathways that may be exploited by the intestinal microbial ecosystem, thereby revealing active metabolic pathways associated with a particular diet and host digestive system physiology combination.

Chapter 4

Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features

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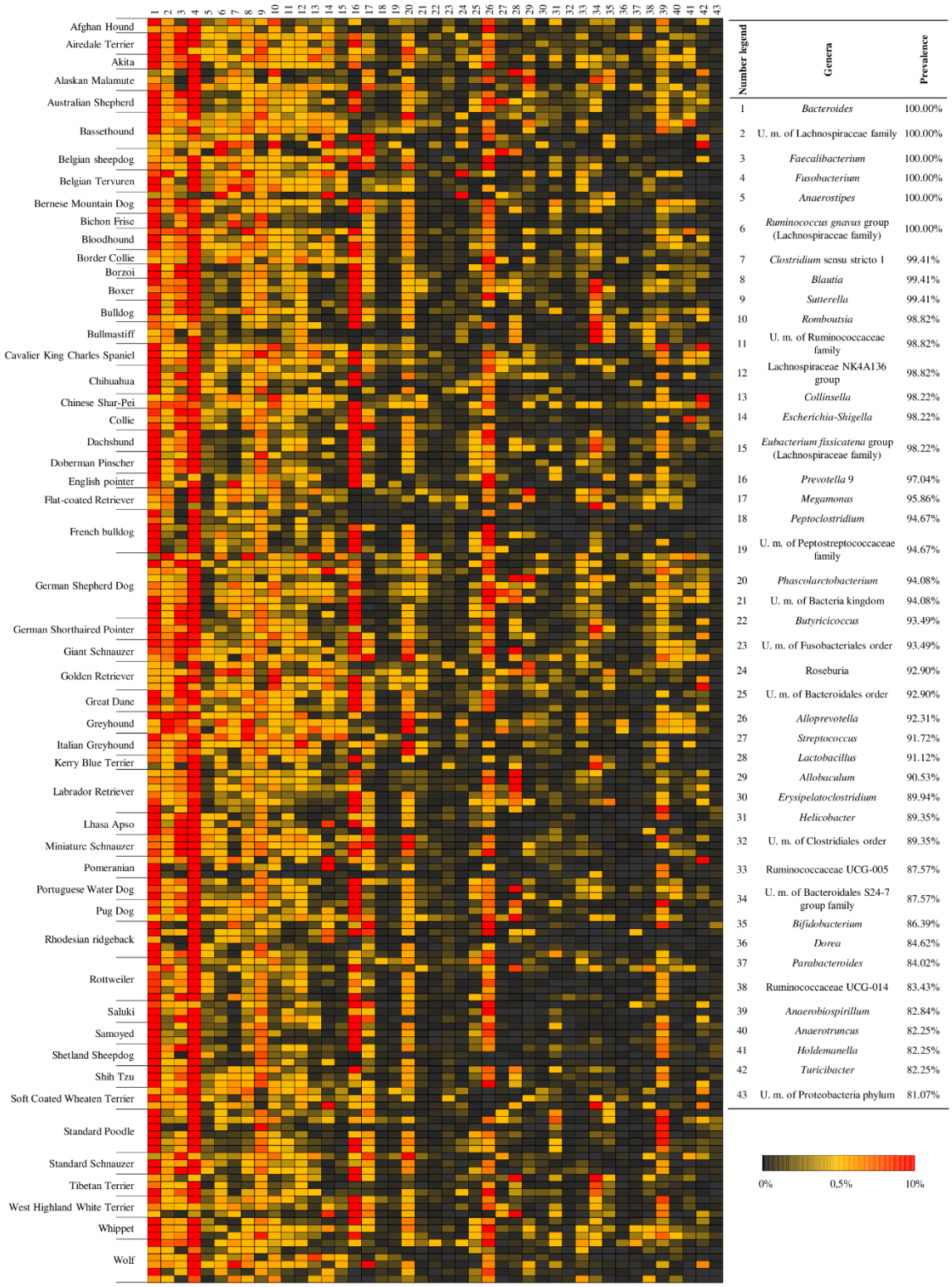
Taxonomic classification of the intestinal microbial community of *Canis lupus*

In order to explore the taxonomical composition of the gut microbiota of the mammalian species *Canis lupus*, a total of 175 fecal samples were collected. In detail, six of these fecal samples belonged to specimens of the grey wolf, while the other 169 fecal samples belonged to members of 51 canine breeds, uniformly distributed along the phylogenetic cluster of breeds as reconstructed by Parker *et al*. based on SNP genotype analysis (Parker *et al.*, 2004). Metadata of these collected samples are reported in Table S1. Bacterial DNA extracted from the fecal samples was subjected to 16S rRNA gene sequencing analysis as previously described (Milani *et al.*, 2013). Illumina-mediated sequencing of the abovementioned samples generated a total of 12,702,820 sequencing reads with an average of 72,588 reads per sample (Supporting Information Table S2). Quality and chimera filtering produced a total of 8,329,451 filtered reads with an average of 47,597 filtered reads per sample (Supporting Information Table S2). Taxonomic reconstruction of the bacterial population encompassed by each of the analysed samples is reported in Supporting Information File 1. Alpha-diversity analysis, performed through Chao1 index calculation for 10 sub-samplings of sequenced read pools, showed that all curves tend to plateau, thereby indicating that sample biodiversity was in all cases adequately covered by the applied sequencing depth (Supporting Information Fig. S1). Moreover, PCoA representation of the unweighted Unifrac distance matrix obtained by analysis of the datasets generated by this study did not reveal any significant clustering as based on evolutionary distance between profiled domesticated dog breeds and their wild (i.e. wolf) relative (Fig. S1). In addition, bioinformatics analyses were performed to evaluate if differences in the canine gut microbiota may be dependent on canine breed. However, these analyses did not reveal any statistically significant differences, suggesting that, in this case, host phylogeny divergence plays a minor role in the modulation of dogs' gut population.

Genus-level core gut microbiota of the *Canis lupus familiaris*

Reconstruction of a core microbiota, which represents bacterial taxa that are shared across samples of a defined cohort (Salonen *et al.*, 2012), allows identification of dominant and prevalent bacterial species that have been preserved during co-evolution of the intestinal community and its host (Tap *et al.*, 2009, Salonen *et al.*, 2012). In order to determine the core gut bacterial community of the collected fecal samples, bacterial genera present in at least 80 % of the samples and with at least an average relative abundance of >0.01 % were considered. Based on these criteria, we identified 43 bacterial genera (Figure 1). In detail, at phylum level the core microbiota was dominated by taxa belonging to Bacteroidetes (total average abundance 33.68 %), followed by Fusobacteria (25.53 %), Firmicutes (23.56 %), Proteobacteria (6.29 %) and Actinobacteria (0.93 %) (Figure 1). As could be expected, the core microbiota includes genera of the five dominant phyla generally found in the canine fecal microbiota (Hand *et al.*, 2013, Moon *et al.*, 2018). Furthermore, at genus level, a particular representative of the Fusobacteria phyla, i.e., *Fusobacterium*, was shown to be present at the highest average relative abundance (25.36 %) among all domesticated dog breeds (Figure 1), suggesting extensive co-evolution between this taxon and the canine GI. Moreover, *Prevotella* 9 and *Bacteroides*, which both belong to the Bacteroidetes phylum, were second and third most abundant genera in the canine microbiota (13.86 % and 13.43 %, respectively). In a human context, *Bacteroides* and *Prevotella* have been linked to a vegan or vegetarian diet (De Filippo *et al.*, 2010). Therefore, the high abundance of these two genera in the canine gut microbiota may be due to the transition from a carnivorous diet typical of wolves to the omnivorous diet of domestic dogs (see below).



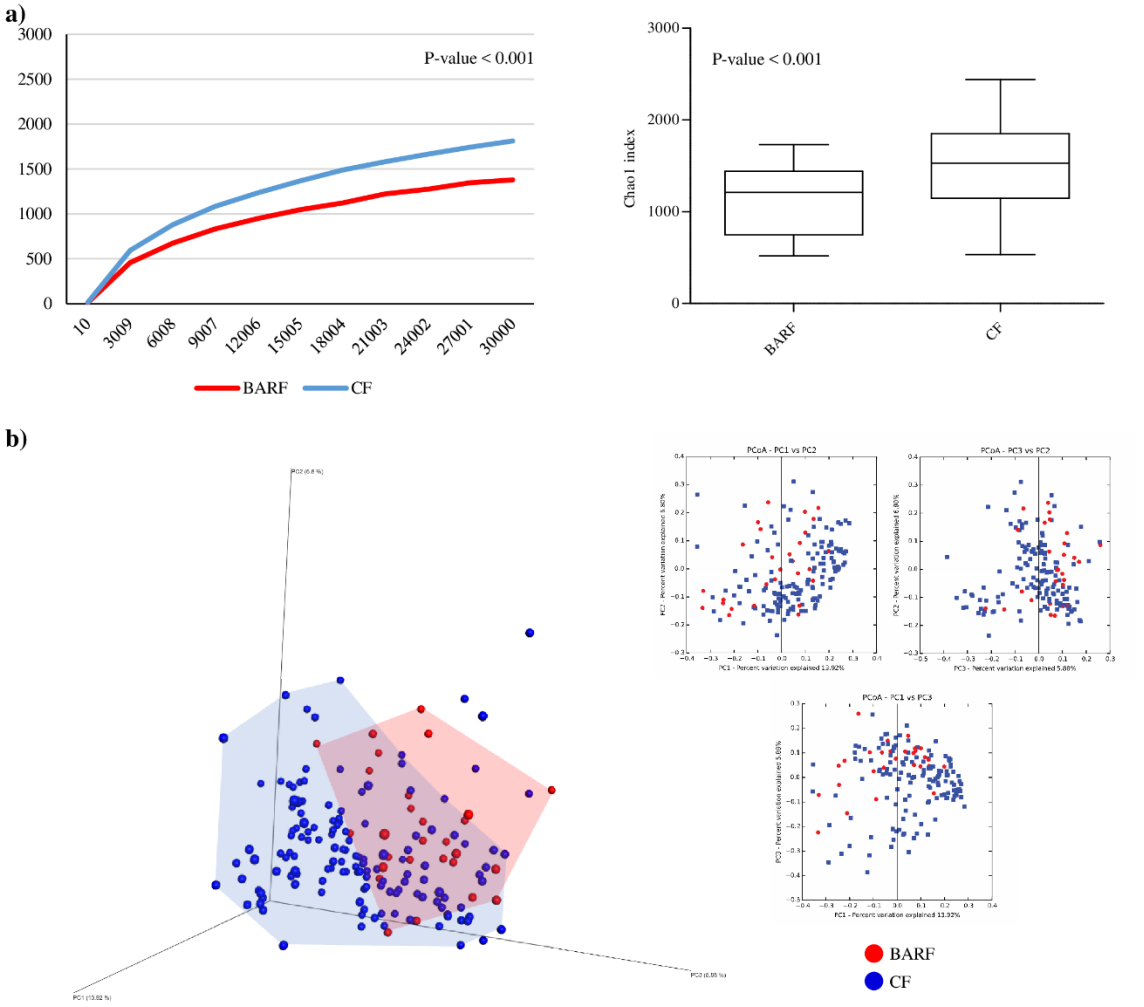
Number legend	Genera	Prevalence
1	*Bacteroides*	100.00%
2	U. m. of Lachnospiraceae family	100.00%
3	*Faecalibacterium*	100.00%
4	*Fusobacterium*	100.00%
5	*Anaerostipes*	100.00%
6	*Ruminococcus gnavus* group (Lachnospiraceae family)	100.00%
7	*Clostridium* sensu stricto 1	99.41%
8	*Blautia*	99.41%
9	*Sutterella*	99.41%
10	*Romboutsia*	98.82%
11	U. m. of Ruminococcaceae family	98.82%
12	Lachnospiraceae NK4A136 group	98.82%
13	*Collinsella*	98.22%
14	*Escherichia-Shigella*	98.22%
15	*Eubacterium fissicatena* group (Lachnospiraceae family)	98.22%
16	*Prevotella* 9	97.04%
17	*Megamonas*	95.86%
18	*Peptoclostridium*	94.67%
19	U. m. of Peptostreptococcaceae family	94.67%
20	*Phascolarctobacterium*	94.08%
21	U. m. of Bacteria kingdom	94.08%
22	*Butyricicoccus*	93.49%
23	U. m. of Fusobacteriales order	93.49%
24	Roseburia	92.90%
25	U. m. of Bacteroidales order	92.90%
26	*Alloprevotella*	92.31%
27	*Streptococcus*	91.72%
28	*Lactobacillus*	91.12%
29	*Allobaculum*	90.53%
30	*Erysipelatoclostridium*	89.94%
31	*Helicobacter*	89.35%
32	U. m. of Clostridiales order	89.35%
33	Ruminococcaceae UCG-005	87.57%
34	U. m. of Bacteroidales S24-7 group family	87.57%
35	*Bifidobacterium*	86.39%
36	*Dorea*	84.62%
37	*Parabacteroides*	84.02%
38	Ruminococcaceae UCG-014	83.43%
39	*Anaerobiospirillum*	82.84%
40	*Anaerotruncus*	82.25%
41	*Holdemanella*	82.25%
42	*Turicibacter*	82.25%
43	U. m. of Proteobacteria phylum	81.07%

Figure1. Taxonomic distribution of the 43 core bacterial genera of the canine gut microbiota. The heat map shows the relative abundance of the 43 bacterial genera that constitute the canine core gut microbiota of the 175 analyzed samples. On the left-hand side, sample breed is reported and samples were ordered as indicated in Supplementary Table S1. In the upper part of the heat map, numbers correspond to the 43 core bacterial genera listed on the right-hand side together with the corresponding prevalencetaxonomic distribution of the 43 core bacterial genera of the canine gut microbiota. the heat map shows

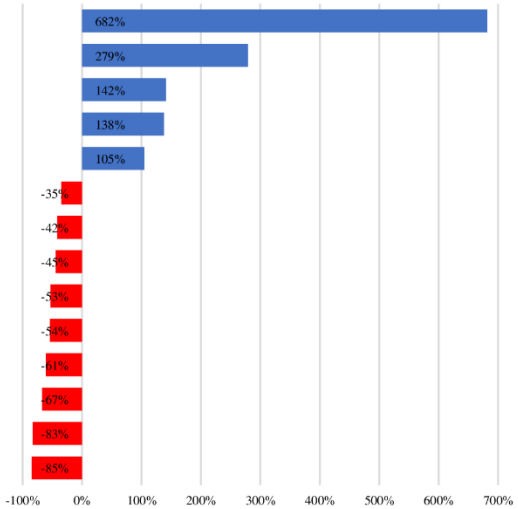
Role of diet as modulator of the canine core gut microbiota

As reported in Supporting Information Table S1, the collected samples belonged to dogs that followed different diets: 141 dogs had been fed with commercial food preparations, typically produced to guarantee a balanced nutritional intake, with a high abundance of fibres and carbohydrates generally higher than 3 % and 30 %, respectively. In contrast, the diet of 28 dogs was based on BARF. Therefore, in order to determine whether and to what extent diet may modulate the canine core gut microbiota, the collected samples were divided into two groups, encompassing dogs fed with commercial food (CF group) and dogs following a BARF diet (BARF group).

Evaluation of the bacterial biodiversity of the two diet groups was performed through the Chao1 index calculated for 10 sub-samplings of sequenced read pools obtained for each sampled dog up to a maximum of 30,000 reads. The two curves, corresponding to the average observed for the CF and BARF groups, are significantly different based on Student's t-test statistical analysis calculated at 30,000 reads (p-value < 0.01) (Fig. 2a). Interestingly, the average rarefaction curves showed a higher level of complexity of the CF group gut microbiota compared to the BARF group. Moreover, the β-diversity was analysed based on unweighted UniFrac and represented through Principal Coordinate Analysis (PCoA) (Fig 2b). The predicted PCoA exhibited partial clustering of CF and BARF groups (P-value < 0.01), supporting the notion that the two distinct diets indeed cause differences in the canine gut microbiota. In addition, analysis of the predicted taxonomic profiles at phylum level revealed that the average abundance of three of the five phyla that are present in the canine core gut microbiota appeared to be altered by diet. Specifically, Fusobacteria and Actinobacteria were significantly increased in dogs fed on a BARF diet, while Bacteroidetes showed an opposite trend (Fig. 2; Table S3). An in-depth inspection at genus level revealed that 14 of the 43 core genera are significantly affected by diet (Fig. 2c). Interestingly, the two most representative genera of the core microbiota, i.e., *Fusobacterium* and *Bacteroides*, did not significantly fluctuate in the two assessed canine groups (Fig. 2c).



c)



Phylum	Genus	BARF	CF	% absolute (CF-BARF)	p-value
Firmicutes	*Faecalibacterium*	0.71%	5.59%	4.88%	0.00005
Bacteroidetes	U. m. of Bacteroidales order	0.08%	0.29%	0.22%	0.02119
Proteobacteria	*Sutterella*	1.28%	3.08%	1.81%	0.00028
Bacteroidetes	*Prevotella* 9	6.44%	15.33%	8.90%	0.00639
Firmicutes	U. m. of Clostridiales order	0.02%	0.04%	0.02%	0.01669
Bacteroidetes	*Alloprevotella*	6.49%	4.23%	-2.27%	0.02399
Firmicutes	*Peptoclostridium*	0.11%	0.06%	-0.05%	0.04151
Firmicutes	U. m. of Peptostreptococcaceae family	0.15%	0.08%	-0.07%	0.04478
Fusobacteria	U. m. of Fusobacteriales order	0.14%	0.07%	-0.08%	0.00675
Actinobacteria	*Collinsella*	1.21%	0.55%	-0.66%	0.02994
Actinobacteria	*Bifidobacterium*	0.52%	0.20%	-0.31%	0.01099
Bacteroidetes	*Parabacteroides*	0.19%	0.06%	-0.13%	0.00600
Firmicutes	*Clostridium* sensu stricto 1	4.10%	0.68%	-3.41%	0.00000
Firmicutes	*Allobaculum*	2.04%	0.31%	-1.73%	0.00002

Figure 2. Evaluation of α- and β- diversity in BARF- and CF-fed dog fecal samples. Panel a shows the representation of α-diversity trough average rarefaction curves on the left-side, and Box and Whisker graphic on the right-side. Average rarefaction curves represent variation of the Chao1 index at increasing sequencing depth of BARF and CF samples. Panel b displays the predicted PCoA encompassing the 169 domesticated canine fecal samples through a three-dimensional image and three two-dimensional sections. Panel c displays the relative abundance variation of significantly different genera between the BARF and CF groups together with their corresponding phylum, absolute percentage and p-value.

Otherwise, *Prevotella 9*, *Faecalibacterium* and *Sutterella* significantly decreased in the BARF group compared to the CF group (Fig. 2c; Supporting Information Table S3). In this context, it has been shown that a high abundance of *Prevotella* in the human gut microbiota correlates with a fiber-based diet, due to the capability of members of this microbial genus to degrade simple carbohydrates (David *et al.*, 2014, Schnorr *et al.*, 2014), while it is known that *Faecalibacterium* spp. and *Sutterella* spp. can also metabolize a wide range of different carbohydrates (Lopez-Siles *et al.*, 2012, Liu *et al.*, 2016). Therefore, dogs fed with commercial pet foods, which are typically enriched in fibers and carbohydrates, are associated with a higher abundance of these saccharolytic species, as compared with dogs of the BARF group whose diet was based on a high abundance of animal proteins and fats. Notably, in humans, *Faecalibacterium*, and in particular *Faecalibacterium prausnitzii*, is associated with a healthy microbiota (Lopez-Siles *et al.*, 2012). Indeed, as a butyrogenic bacterium, this commensal species has been reported to possess anti-inflammatory features and to positively influence the gut physiology (Sokol *et al.*, 2008). In this context, the reduction of *Faecalibacterium* spp. in the BARF group indicates that a meat-based diet is less protective against inflammatory activity in the canine gut.

Effect of artificial selection and close contact with humans on the canine gut microbiota evolution

The dog was the first animal species to be domesticated from wild grey wolves over 15,000 years ago (Savolainen *et al.*, 2002), thus becoming a very coveted companion animal of humans. In this context, it has been demonstrated that man-made selection of canine breeds generated both phenotypic and genotypic changes in dogs (Savolainen *et al.*, 2002). In order to assess if artificial selection and close contact with humans may have impacted on the canine gut microbiota, the latter was compared to the wolf gut bacterial community. Due to the difficulty of collecting feces of wolves living in wild conditions, we were able to retrieve six fecal samples. Thus, it is worth to underline that additional samples may improve accuracy of the comparative analysis. Considering only the bacterial genera

with a prevalence > 80 %, the analysis showed that the wolf gut microbiota consists of 39 bacterial genera, while 43 bacterial taxa were commonly found in all dog samples. Interestingly, *Bacteroides*, U.m. of Lachnospiraceae family, *Faecalibacterium*, *Anaerostipes*, *Fusobacterium* and *Ruminococcus gnavus* group were shared among all investigated dog and wolf samples (Fig. 3). An additional 17 genera were present in all wolf fecal samples and in more than 80 % of the assessed fecal samples from dogs (Fig. 3), suggesting that these 23 bacterial taxa have co-evolved with the species *Canis lupus*, regardless of human intervention. Interestingly, six genera of the core gut microbiota of wolves, i.e., *Alistipes*, *Pseudomonas*, *Slackia*, *Subdoligranulum*, *Eubacterium coprostanoligenes* group and *Barnesiella*, were not represented in the canine core (Fig. 3), suggesting that modifications in the animal lifestyle and the human influence, i.e., domestication, have promoted a modulation of the gut microbiota of dogs when compared to their wild ancestors. In addition, as predators, the diet of wolves is almost exclusively based on raw meat. Comparison of the BARF and CF groups' gut microbiota to that of wolves further support what is reported above, showing a statistically significant progressive increase in the relative abundance of carbohydrate-degrading taxa such as *Prevotella 9* and *Sutterella,* moving from a raw-meat based diet typical of wolves and BARF dogs to a CF diet. (Supporting Information Fig. S2). Conversely, *Parabacteroides* and Ruminococcaceae UCG-005 exhibited an opposite trend, displaying a significant reduction in relative abundance in the CF group as compared to wolves and dogs belonging to the BARF group whose diet is based on raw-meat (Supporting Information Figure S2).

a)

Taxonomy	Human	Dog	Wolf
Bifidobacterium	0.45%	0.25%	0.01
Collinsella	0.16%	0.66%	1.36%
Slackia	-	-	0.09%
Bacteroides	45.23%	13.43%	13.30%
U. m. of Bacteroidales S24-7 group family	-	1.92%	-
Barnesiella	1.35%	-	0.01%
Odoribacter	0.34%	-	-
Parabacteroides	2.73%	0.08%	-
U. m. of Porphyromonadaceae family	0.09%	-	-
Alloprevotella	-	4.60%	6.46%
Prevotella 9	-	13.86%	0.21%
Alistipes	5.34%	-	0.06%
U. m. of Bacteroidales order	0.05%	0.26%	-
Christensenellaceae R-7 group	1.34%	-	-
Clostridium sensu stricto 1	0.18%	1.25%	3.00%
U. m. of Clostridiales vadinBB60 group family	0.25%	-	-
Eubacterium fissicatena group (Lachnospiraceae family)	-	0.27%	0.40%
Eubacterium hallii group	0.07%	-	-
Eubacterium oxidoreducens group	0.02%	-	-
Eubacterium ventriosum group	0.15%	-	-
Ruminococcus gnavus group (Lachnospiraceae family)	-	1.13%	0.77%
Anaerostipes	0.08%	0.40%	0.17%
Blautia	0.72%	1.86%	1.70%
Coprococcus 3	0.19%	-	-
Dorea	0.23%	0.05%	-
Eisenbergiella	1.06%	-	-
Fusicatenibacter	0.31%	-	-
Lachnoclostridium	0.68%	-	-
Lachnospira	3.43%	-	-
Lachnospiraceae ND3007 group	0.14%	-	-
Lachnospiraceae NK4A136 group	0.63%	0.93%	0.83%
Lachnospiraceae NK4B4 group	0.04%	-	-
Lachnospiraceae UCG-004	0.24%	-	-
Lachnospiraceae UCG-010	0.07%	-	-
Pseudobutyrivibrio	1.58%	-	-
Roseburia	3.24%	0.24%	0.10%
U. m. of Lachnospiraceae family	4.66%	1.92%	1.67%
Peptoclostridium	-	0.07%	0.11%
Romboutsia	0.24%	1.55%	1.38%
U. m. of Peptostreptococcaceae family	-	0.09%	0.08%

Taxonomy	Human	Dog	Wolf
U. m. of Ruminococcaceae family	0.32%	0.51%	0.33%
(Eubacterium) coprostanoligenes group	1.22%	-	-
Eubacterium coprostanoligenes group			
Ruminococcaceae family	-	-	0.01%
Anaerotruncus	0.42%	0.20%	0.11%
Butyricicoccus	-	0.06%	0.16%
Faecalibacterium	3.31%	4.78%	1.49%
Flavonifractor	0.17%	-	-
Intestinimonas	0.08%	-	-
Oscillospira	0.76%	-	-
Ruminiclostridium	0.09%	-	-
Ruminiclostridium 5	0.19%	-	-
Ruminiclostridium 9	0.36%	-	-
Ruminococcaceae UCG-002	3.07%	-	-
Ruminococcaceae UCG-005	0.43%	0.23%	0.94%
Ruminococcaceae UCG-013	0.21%	-	-
Ruminococcaceae UCG-014	0.41%	0.16%	0.29%
Ruminococcus 1	0.53%	-	-
Ruminococcus 2	0.73%	-	-
Subdoligranulum	0.79%	-	0.01%
U. m. of Clostridiales order	0.08%	0.04%	-
Allobaculum	-	0.60%	0.36%
Erysipelatoclostridium	-	0.09%	0.05%
Holdemanella	-	0.31%	-
Turicibacter	-	0.71%	-
Lactobacillus	-	2.46%	0.05%
Streptococcus	0.09%	0.40%	-
Phascolarctobacterium	-	2.73%	0.88%
Dialister	0.75%	-	-
Megamonas	-	1.75%	3.25%
Fusobacterium	-	25.36%	27.87%
U. m. of Fusobacteriales order	-	0.08%	0.03%
Anaerobiospirillum	-	1.82%	0.14%
U. m. of Proteobacteria phylum	-	0.03%	
Sutterella	0.69%	2.79%	0.81%
Helicobacter	-	0.26%	0.18%
Bilophila	0.08%	-	-
Escherichia-Shigella	0.82%	1.57%	0.82%
Pseudomonas	-	-	11.71%
U. m. of Bacteria kingdom	0.04%	0.22%	-

b)

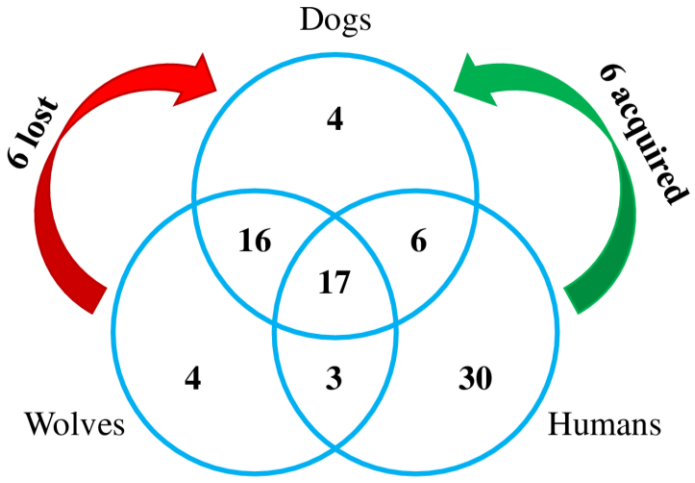


Figure 3. Comparison of core gut microbiota of humans, dogs and wolves. Panel a represents the heat map reporting the bacterial genera that constitute the core gut microbiota of humans, dogs and wolves. The symbol – indicates that the relative bacterial genus is not represented or at least present with a prevalence of < 80 %. Panel b displays the Venn-diagram related to the heat map showing the number of genera that are shared and unique in the core gut microbiota of the three compared mammalian species.

Evaluation of shared and unique bacterial genera of the canine core intestinal community as compared to the human core gut microbiota

To assess if domestication of dogs and their cohabitation with humans has allowed microbiota exchanges, we compared the canine core gut microbiota with that of humans. In order to include comparable 16S rRNA gene microbiota profiling data, the reconstruction of the human core gut microbiota was assessed through the re-analysis of 79 fecal samples of healthy adult individuals used as control group in a previous study where the experimental procedures were the same of this study (Mancabelli *et al.*, 2017). Interestingly, of the six bacterial genera common to all canine samples, *Fusobacterium* and *Ruminococcus gnavus* group were not represented in the human microbiota (Figure 3), indicating that these microbial taxa are typical inhabitants of the canine gut. Moreover, domestication seemed to have caused the loss of six bacterial genera in dogs with respect to its wild relative (Figure 3), while just five microbial taxa were specifically shared between human and canine core gut microbiota (Figure 3). Indeed, *Dorea*, *Parabacteroides*, *Streptococcus*, U. m. of Bacteroidales order and U. m. of Clostridiales order were present in both the human and dog core gut microbiota yet were absent in the core gut microbiota of wolves (Figure 3). These data therefore suggest that the shift from a natural, undomesticated lifestyle to that which involved cohabitation with humans has caused major changes in the bacterial composition of domesticated dogs.

The effect of aging on canine core gut microbiota

Canine life stage classification is known to be affected by both breed and size of dogs (Greer *et al.*, 2007, Fleming *et al.*, 2011, Bartges *et al.*, 2012). In order to evaluate age-related changes in the canine core microbiota, samples were divided into four age groups, while disregarding their breed, including puppies (20 dogs, 0 – 8 months old), junior (27 dogs, 9 – 24 months old), adult (104 dogs, 25 – 96 months old) and senior (18 dogs, > 97 months old). Grey wolves' fecal samples were excluded from this analysis as their age was unknown. Considering the canine core genus microbiota, U. m. of Bacteroidales order, *Phascolarctobacterium*, *Roseburia* and *Fusobacterium* significantly differ

among the four age groups (ANOVA P-value < 0.01) (Fig. S3). Interestingly, a higher level of *Fusobacterium* was reached in canine adulthood (relative abundance 28.80 %), as compared to the junior (20.75 %) and senior (17.58 %) groups. Furthermore, *Roseburia* significantly increased in the senior group, while U. m. of Bacteroidales order was more abundant in puppies when compared to the junior and adult groups. In addition, *Phascolarctobacterium* was shown to be present at a higher abundance in the junior group when compared to the other assessed age groups (Supporting Information Fig. S3)

Moreover, a significant reduction in the abundance of the *Bifidobacterium* genus was apparent in adult and senior groups (average relative abundance of 0.21 % in both cases) when compared to puppies (0.57 %; P-value < 0.05; Supporting Information Fig. S3). Therefore, these data suggest that the bifidobacterial population in the canine gut microbiota exhibits a similar trend to that observed in the human intestinal microbiota (Arboleya *et al.*, 2016, Milani *et al.*, 2017, Turroni *et al.*, 2018).

Profiling of the bifidobacterial community harbored by the canine gut microbiota

In order to further investigate the bifidobacterial communities harbored by the canine gut microbiota, a recently developed pipeline based on genus-specific primers targeting the hypervariable ITS region was applied to all 175 collected samples (Milani *et al.*, 2014). Bifidobacterial ITS microbial profiling produced a total of 12,702,820 reads that were quality-filtered obtaining a total of 8,393,755 reads with an average of 47,964 filtered reads per sample (Supporting Information Table S4). Taxonomic reconstruction of the bifidobacterial population harbored by the analyzed samples is reported in Supporting Information File S2. The obtained ITS data revealed that *Bifidobacterium breve* (25.55 %), *Bifidobacterium pseudolongum* subsp. *globosum* (16.05 %), *Bifidobacterium longum* subsp. *longum* (15.38 %), *Bifidobacterium adolescentis* (9.75 %) and *Bifidobacterium pseudolongum* subsp. *pseudolongum* (6.68 %) were both the most abundant as well as the most prevalent (98.80 %, 99.40 %, 98.80 %, 100 %, 99.40 %, respectively) bifidobacterial taxa in the canine gut microbiota

(Supporting Information Fig. S4). Moreover, seven additional bifidobacterial species, i.e., *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *suis*, *Bifidobacterium dentium*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium magnum*, *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum*, were found to be among the most prevalent (>90 %) canine gut commensals despite showing lower relative abundance (<4.00 %), suggesting their adaptation to colonization of the canine gut. Interestingly, these species were reported to be among the most dominant bifidobacterial taxa in the gut of all mammals (Milani *et al.*, 2017), with the exception of *B. breve*, *B. catenulatum* and *B. pseudocatenulatum.* In fact, the latter species are considered to be more typically associated with the human gut (Turroni *et al.*, 2018). Therefore, the high prevalence of *B. breve*, *B. catenulatum* and *B. pseudocatenulatum* in the canine gut microbiota suggests that human influence, such as artificial selection, dietary contents and subsequent co-habitation of dogs with their owners, may have promoted adaptation and colonization of these bacterial species in the dog GI tract. In this context, comparison of the bifidobacterial population between domesticated dogs and wolves highlighted that the domesticated canine gut is colonized by both a higher average relative abundance (0.25 % and 0.01 % in dogs and wolves, respectively) and a higher level of diversity in terms of number of different bifidobacterial species. Indeed, when considering only known species with an average relative abundance of > 0.01 %, 44 bifidobacterial taxa were detected in the domesticated canine microbiota, while only 14 were found in that of their wild relatives. Moreover, only three of these 14 bifidobacterial taxa, i.e., *B. adolescentis*, *B. longum* subsp. *longum* and *Bifidobacterium dentium* showed a prevalence of > 80 %, thus supporting the notion that a close interaction with humans, a domesticated lifestyle and corresponding diet may have favored horizontal transmission, sub-sequent colonization and persistence of bifidobacterial members from Hominidae to Canidae.

As described above, diet is a contributory factor in modulating the microbial community of the canine gut microbiota. Thus, in order to evaluate the impact that different diets may have on the bifidobacterial population, the obtained ITS sequences of the BARF and CF groups were compared.

Of the five most abundant bifidobacterial species of canine gut microbiota, only *B. pseudolongum* subsp. *pseudolongum* was significantly different between BARF and CF dogs (11.48 % and 5.71 % in BARF and CF groups, respectively, p-value = 0.014). Conversely, *B. breve*, *B. pseudolongum* subsp. *globosum*, *B. longum* subsp. *longum* and *B. adolescentis* showed no significant differences, indicating that these species are resilient to dietary changes, probably due to extensive co-evolution with the host. Nevertheless, the abundance of other, less represented bifidobacterial species appears to be modulated by diet. Indeed, *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium choerinum*, which represent two bifidobacterial species typically found in the mammalian gut, showed an increased relative abundance in the BARF group relative to the CF group. In contrast, *B. catenulatum*, *B. magnum* and *B. pseudocatenulatum* decreased in the BARF group respect to CF group. Interestingly, both *B. animalis* subsp. *animalis* and *B. coherinum* both displayed a prevalence of 100 % in the BARF group, while in the CF group they were prevalent at just 18.84 % and 47.10 %, respectively. The reduced relative abundance and prevalence of these latter taxa points to the possibility that they are selected by a meat-based diet.

Notably, the presence of putative bifidobacterial novel species in the canine gut microbiota was evaluated following the protocol previously described by Milani *et al*. (Milani *et al.*, 2014, Milani *et al.*, 2017). Interestingly, among the detected putative bifidobacterial novel species, one putative new bacterial taxon, previously named new_taxa_43 (Milani *et al.*, 2014), was present at a prevalence of >80 % in both wolves and dogs, suggesting that this new taxon has co-evolved with the *Canis lupus* species.

Functional characterization of the fecal microbiomes of BARF and CF dogs

Shotgun metagenomic data allows the assessment of the metabolic repertoire of an entire complex microbial population through analysis of all coding genes, i.e. the microbiome (Quince *et al.*, 2017). Therefore, in order to evaluate possible differences in the microbiomes of BARF and CF groups, a BARF sample (C99) and a CF sample (C41) were subjected to shotgun metagenomic sequencing.

Selection of these particular two samples was based on the 16S rRNA microbial profiling in order to select the closest canine fecal samples to the average of their corresponding group. Shotgun metagenomic sequencing generated 9,706,454 reads for the CF sample and 13,531,961 reads for the BARF sample, that were then analyzed using the METAnnotatorX software pipeline (Milani *et al.*, 2018).

In silico characterization of putative GHs (Glycosyl Hydrolases), i.e., enzymes that hydrolyze complex carbohydrates into mono- or oligomeric glycan constituents, showed that CF microbiome datasets possessed proportionately more reads classified as GHs (5.31 % and 2.72 % in CF and BARF group, respectively; Supporting Information Table S5). More specifically, genes encoding members of GH families GH2, GH31, GH92 and GH97, which include β-galactosidase, α-glucosidase, α-mannosidase and α- galactosidase activities, respectively, constituted 0.74 % of the CF samples and 0.08 % of the BARF samples (Supporting Information Fig. S4). Similarly, GH families involved in the breakdown of complex polysaccharides derived from plants such as GH3 (L-arabinofuranosidase), GH43 (xylanase), GH51 (endoglucanase)and GH77 (amylomaltase) (Matsuzawa *et al.*, 2015), were more represented in the CF datasets, corresponding with 0.95 % and 0.26 % of the CF and BARF samples, respectively (Supporting Information Fig. S5). These differences may be explained by the increased intake of carbohydrates and fibers of vegetable origin by the CF group (when compared to the BARF group), indicating that the gut microbial glycobiome of dogs is influenced by diet. In parallel, analysis of predicted bacterial metabolic pathways based on MetaCyc classification revealed that genes involved both in amino acid degradation pathways and fatty acid and lipid degradation are more abundant in the BARF sample (Supporting Information Fig. S5), suggesting that an increased animal fat and protein intake favors colonization of the canine gut by microorganisms with an enriched repertoire of amino acid and lipid degradation pathways.

Chapter 5

The impact of human-facilitated selection on the gut microbiota of domesticated mammals

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Effect of domestication on mammalian gut microbiota

In order to investigate the impact of domestication on the mammalian gut microbiota, the fecal bacterial composition of domesticated animals was compared to that of their captivity/wild-living (CWL) counterparts. For this purpose, we collected 146 fecal samples of various mammals, encompassing 94 fecal samples obtained from domesticated mammals (domesticated group) and 52 stool samples retrieved from corresponding CWL species (CWL group), and encompassing 12 domesticated-CWL pairs (Table 1). In this context, because of the extreme difficulty in tracing back fecal samples of CWL animals living in wild conditions, with the exception of wolves, the gut microbiota of domesticated animals was compared to the mammalian species representing the closest relative living in a natural (non-domesticated) environment (Table 1). MiSeq sequencing of the 146 samples produced a total of 8,188,662 sequencing reads with an average of 56,087 reads per sample (Table S1, Supporting Information). After quality and chimera filtering, a total of 7,579,313 filtered reads with an average of 51,913 filtered reads per sample was obtained (Table S1, Supporting Information).

Table 1. List of domesticated-CWL couples involved in the study. The scientific name of each animal species is reported on the left, while the common name is indicated on the right.

Domesticated animals	Feral animals
Bos Taurus (Domestic cow)	*Bos taurus primigenius* (Auroch)
Canis lupus familiaris (Dog)	*Canis lupus* (Wolf)
Capra hircus (Goat)	*Ammotragus lervia* (Barbary sheep)
Equus asinus (Domestic donkey)	*Equus africanus* (Wild donkey)
Equus ferus caballus (Horse)	*Equus ferus* (Wild horse)
Felis silvestris catus (Cat)	*Felis silvestris* (Wild cat)
Lama glama (Llama)	*Lama guanicoe* (Guanaco)
Mus musculus (House mouse)	*Apodemus sylvaticus* (Wood mouse)
Oryctolagus cuniculus (European rabbit)	*Lepus europaeus* (European hare)
Ovis aries (Domestic sheep)	*Ovis musimon* (Mouflon)
Sus scrofa domesticus (Domestic pig)	*Sus scrofa* (Wild boar)
Vicugna pacos (Alpaca)	*Vicugna vicugna* (Vicuna)

Exploration of the bacterial biodiversity (alpha-diversity) based on Chao1, Shannon and phylogenetic diversity indexes as well as on observed OTUs highlighted that in all cases rarefaction curves tend to reach the plateau for each of the 146 mammalian fecal samples (Fig. S1, Supporting Information). Moreover, rarefaction curves generated by the calculation of Good's coverage index tend to reach the value 1 (Fig. S1, Supporting Information). Altogether these results suggest that the obtained sequencing data was adequate to cover most of the microbial complexity present in the collected samples (Fig. S1 and Excel file S1, Supporting Information). Furthermore, taxonomic data were used to evaluate beta-diversity by means of principal coordinate analysis. In this context, ANOSIM statistics revealed a statistically significant difference between the fecal microbiota composition of the domesticated group when compared to CWL animals (p-value = 0.043 and R-value = 0.517) (Fig. S2, Supporting Information), suggesting that domestication has contributed to a modulation of the taxonomical composition of mammalian fecal microbial population, when disregarding minor intergroup variabilities. Furthermore, analysis of the average phylum-level taxonomic profiles of the CWL and domesticated groups highlighted that Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria were the most abundant and the most prevalent phyla in both domesticated and CWL animals (Table 2 and Excel File S1, Supporting Information). However, the Proteobacteria phylum showed a significantly higher average relative abundance in samples from the CWL group when compared to those from their domesticated relatives (16.98 % ± 3.79 % and 6.10 % ± 3.79 %, respectively) (Student's t-test p-value < 0.01) (Table 2 and Excel File 1, Supporting Information). Furthermore, the Fusobacteria phylum was present at a higher average relative abundance in domesticated mammals (5.93 % ± 1.66 % and 3.86 % ± 1.66 % in the domesticated and CWL groups, respectively), but at a lower prevalence compared to the other four major phyla (Table 2 and Excel File S1, Supporting Information). Indeed, the Fusobacteria phylum was predominantly found in fecal samples of carnivores (dogs and cats) (Excel File 1, Supporting Information).

Table 2. Bacterial phyla with an average relative abundance of ≥ 0.01 in at least one of the two compared groups (domesticated vs. CWL groups). All values, except for those reported in the p-value column, are indicated as percentage. The p-value of bacterial phyla that statistically differ between domesticated and CWL groups are written in bold. Legend: SD = Standard Deviation, SEM = Standard Error Mean.

Phylum	Average domesticated	Average CWL	Prevalence domesticated	Prevalence CWL	SD domesticated	SD CWL	SEM domesticated	SEM CWL	p-value	Benjamini-Hochberg Adjusted p-value	Reject null hypothesis
Firmicutes	52.49	49.09	100.00	100.00	21.73	27.64	2.24	3.83	0.413	0.551	FALSE
Bacteroidetes	28.27	23.84	100.00	100.00	17.10	15.78	1.76	2.19	0.126	0.420	FALSE
Proteobacteria	6.10	16.98	100.00	100.00	12.10	27.32	1.25	3.79	**0.001**	**0.023**	FALSE
Fusobacteria	5.93	3.86	48.94	30.77	13.56	11.95	1.40	1.66	0.359	0.598	FALSE
Actinobacteria	1.86	1.57	97.87	98.08	3.67	3.08	0.38	0.43	0.629	0.740	FALSE
Verrucomicrobia	1.12	0.48	70.21	59.62	2.22	1.19	0.23	0.17	0.055	0.550	FALSE
Spirochaetes	0.89	1.26	64.89	69.23	1.70	2.33	0.18	0.32	0.273	0.545	FALSE
U. m. of Bacteria kingdom	0.70	0.67	89.36	90.38	0.74	0.90	0.08	0.12	0.834	0.834	FALSE
Tenericutes	0.69	0.55	80.85	82.69	0.75	0.70	0.08	0.10	0.279	0.507	FALSE
Fibrobacteres	0.44	0.12	40.43	26.92	1.42	0.45	0.15	0.06	0.118	0.471	FALSE
Kiritimatiellaeota	0.41	0.29	50.00	48.08	0.75	0.84	0.08	0.12	0.397	0.567	FALSE
Epsilonbacteraeota	0.37	0.25	71.28	61.54	0.93	0.62	0.10	0.09	0.385	0.592	FALSE
Cyanobacteria	0.26	0.45	77.66	78.85	0.37	0.84	0.04	0.12	0.056	0.376	FALSE
Patescibacteria	0.19	0.38	64.89	57.69	0.27	0.92	0.03	0.13	0.068	0.338	FALSE
Lentisphaerae	0.15	0.06	46.81	36.54	0.45	0.14	0.05	0.02	0.140	0.350	FALSE
Planctomycetes	0.04	0.02	42.55	34.62	0.17	0.04	0.02	0.01	0.450	0.562	FALSE
Synergistetes	0.03	0.05	26.60	34.62	0.08	0.10	0.01	0.01	0.254	0.565	FALSE
Deferribacteres	0.03	0.02	13.83	11.54	0.20	0.11	0.02	0.01	0.806	0.848	FALSE
Elusimicrobia	0.02	0.02	38.30	19.23	0.05	0.07	0.01	0.01	0.745	0.827	FALSE
Acidobacteria	0.00	0.03	5.32	7.69	0.00	0.19	0.00	0.03	0.132	0.377	FALSE

Further detailed assessment of the taxonomic profiles at genus level revealed that six taxa, encompassing U. m. (unclassified member) of Lachnospiraceae family, U. m. of Bacteroidales order, *Bacteroides*, U. m. of Ruminococcaceae family, *Ruminococcaceae* UCG-014 and *Eubacterium coprostanoligenes* group showed a prevalence of >80 % in both domesticated and CWL groups (Table 3 and Excel File 1, Supporting Information), suggesting that these genera have co-evolved with their hosts, regardless of any human influence. Moreover, 12 bacterial genera, including *Ruminococcaceae* UCG-010 (t-test p-value = 0.019, FDR = 0.036, ANCOM = false), *Akkermansia* (t-test p-value = 0.049, FDR = 0.050, ANCOM = false), and *Prevotella* 9 (t-test p-value =0.002, FDR = 0.059, ANCOM = true), showed significantly higher relative abundance in domesticated animals compared to their CWL counterparts (Table 3 and Excel File S1, Supporting Information). Notably, the high abundance of *Prevotella* in the human gut microbiota and the high abundance of *Ruminococcaceae* in animals has been associated with a fiber-based diet since species of these microbial taxa are able to degrade a wide range of carbohydrates (De Filippo *et al.*, 2010, Wu *et al.*,

2011, Menni *et al.*, 2017). In this context, the greater variety of fibers and carbohydrates typical of industrial feeds (Wanapat *et al.*, 2015) may have promoted the colonization of *Prevotella* 9 and *Ruminococcaceae* UCG-010 in the domesticated species' intestinal population, as opposed to the CWL animals whose fiber and carbohydrate intake is restricted to what can be harvested from the natural environment. Moreover, in humans, *Akkermansia* and in particular *Akkermansia muciniphila* has an important role in the maintenance of intestinal homeostasis and is considered to be a marker of healthy gut microbiota (Belzer & de Vos, 2012, Lopez-Siles *et al.*, 2018). Therefore, close contact with humans and subsequent increase in animal care may have favored the colonization of bacterial biomarkers of health in the domesticated animals' gut microbiota as compared to that of their CWL relatives. In contrast, 26 microbial taxa were significantly more represented in the CWL animals compared to their domesticated counterparts (p-value < 0.05) (Table 3 and Excel File S1, Supporting Information). Interestingly, among the latter bacterial genera, some typical environmental bacteria were found, such as *Sporosarcina*, *Chryseobacterium*, U. m. of Sphingomonadaceae family, *Massilia, Pedobacter*, *Flavobacterium* and *Stenotrophomonas* (Gudeta *et al.*, 2016, Jimenez *et al.*, 2016, Sun *et al.*, 2017), suggesting that close contact of CWL animals with natural environments may have promoted the increased abundance of these transient bacterial taxa in the CWL animal gut.

Table 3. Bacterial genera that statistically differ between domesticated and CWL mammals. All values, except for those reported in t-test p-value < 0.05, Benjamini-Hochberg Adjusted p-value and Rejected null hypothesis column, are expressed as percentage. Legend: SD = Standard Deviation, SEM = Standard Error Mean.

Genus	Average domesticated	Average CWL	Prevalence domesticated	Prevalence CWL	SD domesticated	SD CWL	SEM domesticated	SEM CWL	t-test p-value < 0.05	Benjamini-Hochberg Adjusted p-value	Reject null hypothesis
Arthrobacter	0.00	0.07	1.06	13.46	0.00	0.24	0.00	0.03	0.006	**0.036**	FALSE
U. m. of Muribaculaceae family	1.07	2.89	81.91	69.23	3.15	7.54	0.32	1.05	0.042	0.051	FALSE
Prevotella 9	5.26	0.12	48.94	19.23	11.85	0.59	1.22	0.08	0.002	0.059	**TRUE**
Millionella	0.00	0.00	0.00	7.69	0.00	0.02	0.00	0.00	0.014	**0.045**	FALSE
Cytophaga	0.00	0.00	0.00	5.77	0.00	0.01	0.00	0.00	0.040	**0.049**	FALSE
Hymenobacter	0.00	0.04	0.00	11.54	0.00	0.17	0.00	0.02	0.031	**0.046**	FALSE
U. m. of Microscillaceae family	0.00	0.00	0.00	5.77	0.00	0.01	0.00	0.00	0.023	**0.040**	FALSE

Dyadobacter	0.00	0.04	1.06	11.54	0.00	0.16	0.00	0.02	0.014	**0.049**	FALSE
Flavobacterium	0.07	1.29	10.64	23.08	0.63	4.89	0.06	0.68	0.019	**0.040**	FALSE
Chryseobacterium	0.01	0.19	17.02	28.85	0.06	0.57	0.01	0.08	0.003	0.054	FALSE
Mucilaginibacter	0.00	0.01	0.00	7.69	0.00	0.07	0.00	0.01	0.050	**0.050**	FALSE
Pedobacter	0.01	1.18	7.45	21.15	0.07	3.85	0.01	0.53	0.004	**0.029**	FALSE
Sporosarcina	0.01	0.16	8.51	11.54	0.04	0.70	0.00	0.10	0.038	**0.048**	FALSE
U. m. of Bacillales order	0.00	0.02	6.38	11.54	0.02	0.06	0.00	0.01	0.047	0.051	FALSE
Carnobacterium	0.02	0.36	11.70	19.23	0.07	1.36	0.01	0.19	0.017	**0.039**	FALSE
Vagococcus	0.00	0.02	2.13	9.62	0.01	0.08	0.00	0.01	0.019	**0.039**	FALSE
Streptococcus	0.23	0.06	60.64	53.85	0.52	0.16	0.05	0.02	0.025	**0.041**	FALSE
Clostridium sensu stricto 13	0.00	0.00	2.13	11.54	0.00	0.01	0.00	0.00	0.030	**0.047**	FALSE
Peptococcus	0.15	0.07	58.51	46.15	0.24	0.15	0.02	0.02	0.019	**0.035**	FALSE
CAG-352	0.00	0.01	4.26	9.62	0.01	0.04	0.00	0.01	0.037	**0.048**	FALSE
Flavonifractor	0.01	0.22	6.38	15.38	0.07	0.67	0.01	0.09	0.003	**0.042**	FALSE
Intestinimonas	0.00	0.06	4.26	11.54	0.01	0.24	0.00	0.03	0.019	**0.038**	FALSE
Ruminiclostridium 9	0.06	0.25	37.23	42.31	0.17	0.52	0.02	0.07	0.001	0.063	FALSE
Ruminococcaceae UCG-004	0.01	0.06	5.32	15.38	0.05	0.21	0.00	0.03	0.012	**0.047**	FALSE
Ruminococcaceae UCG-008	0.02	0.15	20.21	21.15	0.10	0.40	0.01	0.06	0.004	**0.039**	FALSE
Ruminococcaceae UCG-010	3.13	1.49	69.15	63.46	4.55	2.61	0.47	0.36	0.019	**0.036**	FALSE
U. m. of Clostridia class	0.01	0.00	24.47	3.85	0.02	0.00	0.00	0.00	0.014	**0.047**	FALSE
Holdemanella	0.11	0.00	35.11	5.77	0.34	0.03	0.04	0.00	0.033	**0.047**	**TRUE**
Phascolarctobacterium	0.73	0.46	90.43	57.69	0.87	0.64	0.09	0.09	0.046	0.051	**TRUE**
Gemmatimonas	0.00	0.00	0.00	5.77	0.00	0.01	0.00	0.00	0.030	**0.046**	FALSE
U. m. of Caulobacteraceae family	0.01	0.21	10.64	26.92	0.03	0.93	0.00	0.13	0.037	**0.049**	FALSE
U. m. of Beijerinckiaceae family	0.00	0.00	0.00	7.69	0.00	0.01	0.00	0.00	0.016	**0.042**	FALSE
U. m. of Devosiaceae family	0.00	0.05	5.32	15.38	0.02	0.16	0.00	0.02	0.009	**0.041**	FALSE
U. m. of Sphingomonadaceae family	0.01	0.25	17.02	36.54	0.03	0.96	0.00	0.13	0.016	**0.040**	FALSE
U. m. of Desulfovibrionaceae family	0.03	0.01	39.36	21.15	0.06	0.02	0.01	0.00	0.023	**0.041**	FALSE
Janthinobacterium	0.00	0.18	1.06	11.54	0.03	0.60	0.00	0.08	0.005	**0.036**	FALSE
Massilia	0.00	0.08	2.13	7.69	0.01	0.29	0.00	0.04	0.016	**0.042**	FALSE
Sutterella	0.58	0.13	39.36	28.85	1.39	0.39	0.14	0.05	0.024	**0.040**	FALSE
U. m. of Burkholderiaceae family	0.13	2.09	62.77	65.38	0.28	7.10	0.03	0.98	0.008	**0.039**	FALSE
U. m. of Methylophilaceae family	0.00	0.00	1.06	5.77	0.00	0.02	0.00	0.00	0.048	0.050	FALSE
Cellvibrio	0.00	0.01	0.00	9.62	0.00	0.06	0.00	0.01	0.043	0.050	FALSE
Psychrobacter	0.28	2.55	17.02	19.23	1.57	10.64	0.16	1.48	0.043	**0.048**	FALSE
Pseudomonas	0.65	5.03	34.04	51.92	4.19	14.57	0.43	2.02	0.007	**0.040**	FALSE
U. m. of Gammaproteobacteria class	0.58	0.05	50.00	28.85	1.80	0.16	0.19	0.02	0.034	**0.047**	**TRUE**
Stenotrophomonas	0.00	0.09	8.51	19.23	0.01	0.41	0.00	0.06	0.035	**0.047**	FALSE
Xanthomonas	0.00	0.00	0.00	9.62	0.00	0.01	0.00	0.00	0.004	**0.033**	FALSE
Pyramidobacter	0.00	0.02	10.64	23.08	0.01	0.07	0.00	0.01	0.009	**0.038**	FALSE
U. m. of Mollicutes class	0.03	0.00	18.09	5.77	0.08	0.02	0.01	0.00	0.048	0.050	**TRUE**
U. m. of Izimaplasmatales order	0.13	0.03	47.87	38.46	0.30	0.05	0.03	0.01	0.015	**0.043**	FALSE
U. m. of Mollicutes class	0.00	0.00	15.96	0.00	0.01	0.00	0.00	0.00	0.017	**0.038**	FALSE
U. m. of Puniceicoccaceae family	0.01	0.00	28.72	15.38	0.01	0.01	0.00	0.00	0.033	**0.048**	FALSE
Akkermansia	1.10	0.44	69.15	48.08	2.21	1.19	0.23	0.17	0.049	0.050	FALSE
Luteolibacter	0.00	0.02	2.13	9.62	0.00	0.07	0.00	0.01	0.043	**0.049**	FALSE

*U. m. = Unclassified member

Evaluation of shared and unique bacterial genera during the mammalian evolution

In order to evaluate the precise impact of domestication on the mammalian gut microbiota, we assessed possible loss and acquisition events of bacterial taxa in CWL vs domesticated animals. However, due to difficulties in collecting fecal samples from certain CWL species, only six domesticated-CWL pairs could be evaluated (Table S2, Supporting Information). Moreover, only those bacterial genera that showed a prevalence of ≥80 % and an average relative abundance of ≥0.01 % were included. Overall, for each single pair of animals, shared bacterial genera (ranging from 6 to 53) were observed, indicating that these microbial taxa have co-evolved with their hosts, regardless of domestication. Furthermore, unique bacterial genera were also detected in each domesticated-CWL pair, suggesting that modifications in animal lifestyle and/or human influence have contributed to the modulation of the domesticated animal gut microbiota when compared to its CWL counterpart, through loss and acquisition of microbial taxa (Table S2, Supporting Information). In this regard, some domesticated animals seemed to have acquired specific gut bacterial genera that have been linked to a more efficient conversion of feed into body mass, including *Mitsuokella* (Tan et al., 2018) and Prevotellaceae NK3B31 group (Quan *et al.*, 2018) in porcine fecal microbiota as well as the amylolytic *Saccharofermentans* in both goat and sheep fecal population (Perea *et al.*, 2017) (Table S2, Supporting Information). Moreover, apparent acquisition of bacterial taxa that have evolved specialized systems to degrade complex polysaccharides, such as representatives of the Ruminococcaceae and/or Prevotellaceae families, was observed in the domesticated fecal microbiota when compared to the CWL one, for every domesticated-CWL pair (Flint *et al.*, 2012, Crost *et al.*, 2018) (Table S2, Supporting Information). Notably, efficient degradation of complex carbohydrates allows accumulation of simple glycans in the intestinal tract that can be adsorbed by the host or fermented into short chain fatty acids that can be readily used as an energy source by colonic enterocytes and other tissue cells (Agans *et al.*, 2018). Therefore, these observations suggest that domestication has contributed to the selection of farm animals harboring and supporting colonization

of gut bacteria that maximize energy recovery from feeds in the livestock industry, in order to facilitate rapid increase in animal body weight and improving livestock performances.

At the same time, despite their carnivorous classification, dogs acquired certain saccharolytic species such as *Prevotella* 9 and *Lactobacillus* able to degrade various carbohydrates (Table S2, Supporting Information) (David *et al.*, 2014, Alessandri *et al.*, 2019). Notably, commercial pet foods are produced to guarantee a balanced nutritional intake with an enrichment in fibers and carbohydrates as compared to the raw meat-based diet of their CWL relatives (wolves) (Alessandri *et al.*, 2019). Therefore, changes in the canine diet caused by domestication and human intervention may have promoted modulation of the associated gut microbiota, favouring colonization by the above-mentioned saccharolytic taxa. In addition, analysis of the four herbivorous domesticated-CWL pairs involved in this analysis revealed the presence of U. m. of *Ruminococcaceae* family, U. m. of *Lachnospiraceae* family and *Ruminococcaceae* UCG-014 bacterial genera in the domesticated gut microbial population, in support of the notion that the domestication process played a defining role in colonization of these genera in the intestinal tract of herbivores (Table S2, Supporting Information). Altogether, these findings indicate that the domestication process has led to considerable modulation of the composition of the mammalian gut microbiota, with an associated impact on its metabolic potential (see later).

Effect of anthropogenic influences on the domesticated mammalian gut microbiota

In order to explore the impact of close contact with humans on mammalian gut microbiota composition, the fecal microbial population of domesticated animals was compared to those of both their CWL counterparts and humans. For this purpose, we re-analyzed the 16S rRNA gene microbial profiling data of 79 fecal samples belonging to healthy adult individuals sequenced in a previous study following the same experimental procedures (Mancabelli *et al.*, 2017). In this case, in order to obtain an initial insight in the loss and acquisition of bacterial genera during domestication, such comparisons were restricted to bacterial genera that displayed a prevalence of at least 80 %, in order

to include only those taxa that apparently had been preserved during co-evolution of gut microbiota and its hosts. Notably, diet plays a major role in influencing the gut microbiota composition along the mammalian tree of life (Ley *et al.*, 2008, Finlayson-Trick *et al.*, 2017). Therefore, the two macro-groups, i.e. domesticated and CWL mammalian groups, were sub-divided in herbivores, carnivores and omnivores, obtaining a total of six groups in the comparison to human fecal microbiota (Table S1, Supporting Information). Interestingly, this analysis highlighted that the human gut microbiota shares a higher number of bacterial genera with the herbivore groups, followed by the omnivore and carnivore ones (12, nine and three shared bacterial genera, respectively) (Figure 1). However, for all these three diet-based groups, the human gut microbiota was shown to share a higher number of bacterial taxa with the domesticated mammalian fecal population (7, 20 and 14 shared bacterial genera between humans and domesticated carnivores, omnivores and herbivores, respectively) when compared to the number of microbial genera shared with the CWL groups (Figure 1). Therefore, this observation suggests that interaction with humans allows microbial exchange events between humans and domesticated animals through horizontal transmission. Consistent with such a notion, it was found that carnivorous and omnivorous domesticated animals' gut microbial community shared a higher number of bacterial genera with the human gut microbiota when compared to the respective CWL groups (6 and 19 shared bacterial genera between domesticated omnivores and carnivores and their respective CWL groups, respectively) (Figure 1). This finding is probably due to the fact that carnivore and omnivore diets better resemble the human diet, leading to the selection of a similar microbial gut population and favouring inter-species transmission of bacterial taxa. Therefore, all together, these observations support the idea that the domestication process as imposed by humans, including the modification of animal diet, habitat and behavior, has affected the mammalian gut microbial composition through horizontal acquisition/loss of specific bacterial taxa.

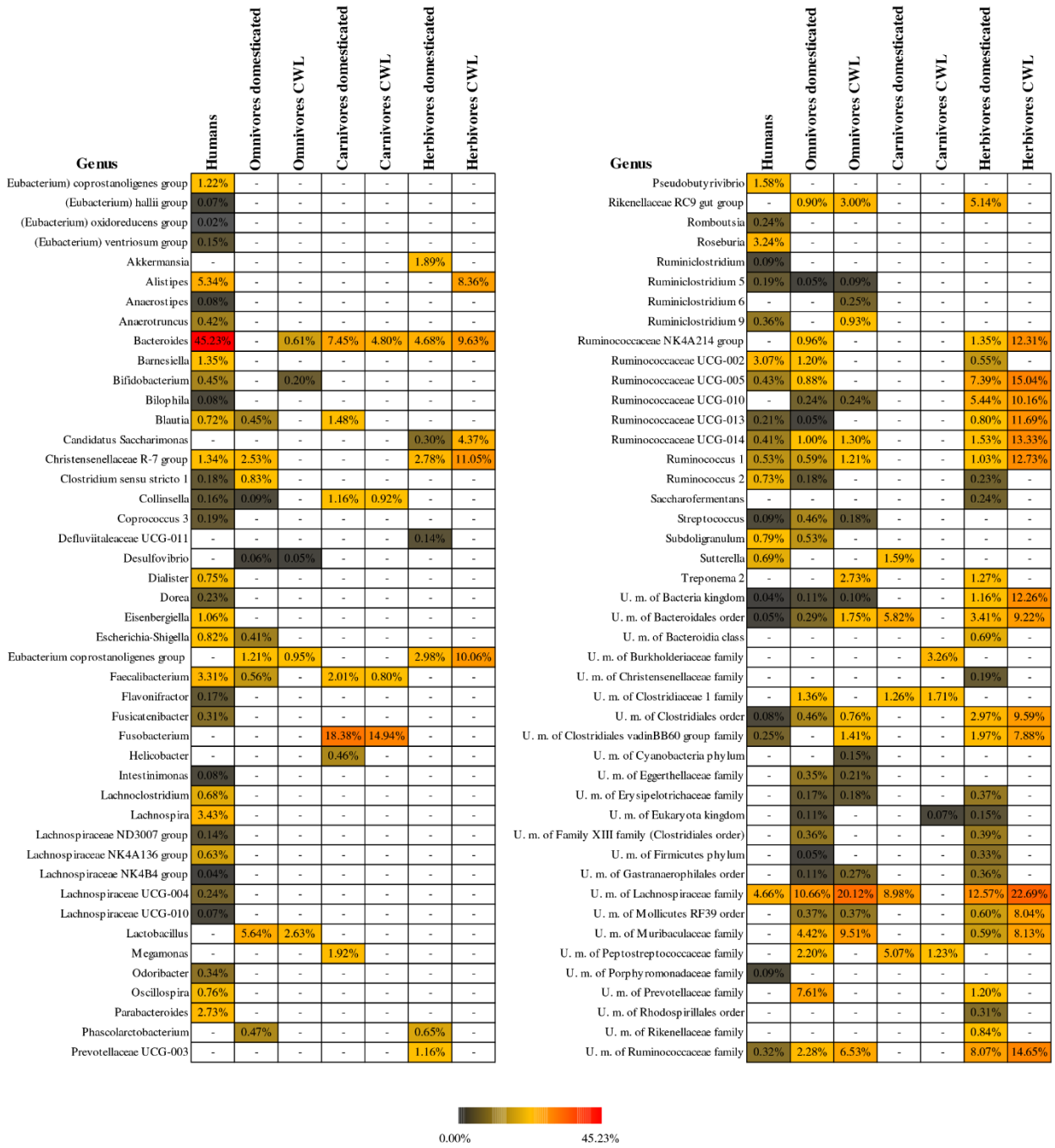

Figure 1. Evaluation of unique and shared bacterial genera of the gut microbiota of seven sub-groups: humans together with domesticated and CWL animals, divided into carnivores, herbivores and omnivores. The heat map shows the average relative abundance of those bacterial genera that were determined to be present at a prevalence of >80 % for each analyzed group.

Functional characterization of the fecal microbiomes of domesticated and CWL groups

In order to obtain insights into the changes in gut microbiota functionality linked to the aforementioned modulation of the taxonomic composition induced by human intervention, a shotgun metagenomic approach was performed for fecal samples of 14 mammalian species, equally divided between domesticated animals and those of their CWL counterparts (Table S3, Supporting Information). Selection of these samples was performed randomly for each species. Shotgun metagenomic sequencing produced a total of 155,850,275 raw reads with an average of 9,740,642 per sample (Table S3, Supporting Information). Due to the putative presence of eukaryotic DNA that may cause biases in the analyses, datasets were mapped to a database containing all available relevant host genomes. Unmapped reads were then filtered by quality, generating a total of 137,886,639 filtered reads with an average of 9,849,046 reads per sample (Table S3, Supporting Information). Subsequently, in order to obtain a functional overview, the 14 microbiomes were subjected to pathway prediction based on the MetaCyc database (Caspi *et al.*, 2016) and glycobiome profiling based on the CAZy database (Lombard *et al.*, 2014).

Comparative analysis of bacterial pathways encoded by the 14 microbiomes analyzed, based on the MetaCyc database, revealed that domestication is associated with a trend of reduction of the total number of pathways constituted by >0.001 % of the total read pool, as observed in six of the seven analyzed cases (Fig. S3, Supporting Information). The same trend was observed when focusing on the number of pathways classified as the 17 main degradative pathway families defined by the MetaCyc database (Fig. S4, Supporting Information). Interestingly, these data suggest that domestication induced a simplification of the overall genetic and functional potential of the gut microbial population of the studied mammals, with the exception of horses. In this context, four pathways were observed to decrease in abundance in all analyzed domesticated species when compared to their CWL counterparts (Fig. S4, Supporting Information), including the pathway phenol degradation II organosulfonate compounds. This latter observation may reflect a reduction in

aromatic compounds, widely distributed in nature as lignin components, flavonoids and quinones, in the diet of domesticated animals (Carmona *et al.*, 2009, Diaz Heijtz *et al.*, 2011).

In contrast, six specific pathways were observed to increase in abundance in domesticated mammals (Fig. S4, Supporting Information). These include the pyruvate fermentation to acetate IV pathway and the super pathway of acetate utilization and formation (Fig. S4, Supporting Information), indicating an increased capability to use pyruvate as an energy source that may be due to increased dietary intake of polysaccharides in domesticated animals (Wanapat *et al.*, 2015). Moreover, domesticated mammals showed higher abundance of reads corresponding to the 4-nitrobenzoate degradation pathway (Fig. S4, Supporting Information), which is involved in the metabolism of nitroaromatic compounds as an energy source. Notably, the latter are toxic compounds widely used for the synthesis of pesticides, plasticizers, dyes as well as pharmaceuticals and are contaminants of food and water (Leuenberger *et al.*, 1988). Close contact of mammals with humans may have increased their interaction with such substances, thus driving adaptation of domesticated mammalian gut microbiota toward detoxification with concomitant energy extraction from nitroaromatic compounds.

Shotgun metagenomic reads were also exploited to reconstruct the glycobiome of domesticated and CWL mammals, revealing that, despite the apparent decrease of the number of metabolic pathways encoded by the domesticated gut microbiomes, the total number of reads being functionally assigned to the glycobiome is higher in domesticated animals than that of their CWL species in six of the seven pairs analyzed (Figure 2).

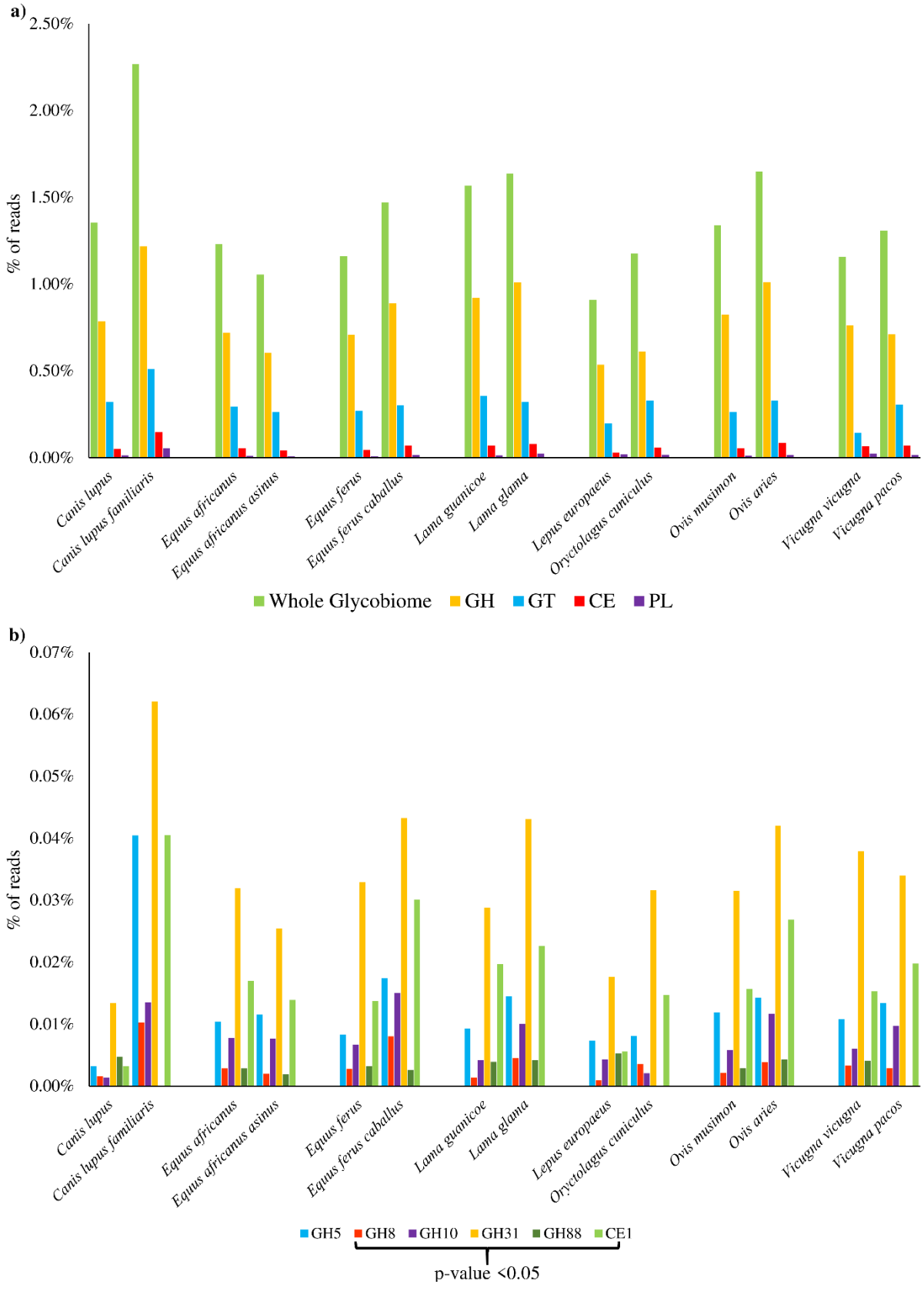

Figure 2. Evaluation of the glycobiome repertoire in the domesticated and CWL microbiome. Panel a shows the percentage of total reads classified as enzymes of the glycobiome and the percentage of reads belonging to the four glyco-active families: GH (Glycoside Hydrolase), GT (Glycosyl Tranferases), CE (Carbohydrate esterases) and PL (Polysaccharide lyases). Panel b displays the GH families that statistically differ between the domesticated and feral gut microbiome.

Notably, these data indicate that the gut microbiomes of domesticated mammals are functionally more focused on the metabolism of complex sugars. More specifically, Student's t-test statistical analysis of all enzyme classes recognized by the CAZy database revealed that the reads classified as carbohydrate esterase family 1 as well as glycoside hydrolases (GH) families 8, 10 and 31 exhibit a statistically significant increase in domesticated mammals (Student's t-test p-value <0.05), ranging from 45 % to 137 %, while GH88 showed a decrease of -52 % (Student's t-test p-value <0.05) (Figure 2). Moreover, GH5 was observed to increase in all domesticated species, with such increases ranging from 10 % to 1182 %. Nevertheless, Student's t-test provided a *p*-value of 0.075 (Figure 2). Intriguingly, GH31 encompasses α-glucosidases, which is the primary enzyme involved in the degradation of starch. Moreover, enzymes classified as GH5, GH8 as well GH10 participate in the breakdown of xylan and xylooligosaccharides, such as arabinoxylans, while GH5 is also involved in the metabolism of cellulose. Notably, the increased abundance of these glycosyl hydrolases in the microbiome of domesticated mammals indicates a specific functional adaptation of their gut microbiota to the higher intake of these complex plant carbohydrates as compared to their CWL equivalents. This is probably explained by the extensive use of legumes (rich in dietary fibers such as resistant starch and cellulose) and whole grains (rich in bran) as main components of animal feeds (Wanapat *et al.*, 2015).

Effect of domestication on the bifidobacterial community of the mammalian gut

Bifidobacterial species are known to be common components of the mammalian gut microbiota (Milani *et al.*, 2017). In order to provide an in-depth view of the impact that domestication may have exerted on the mammalian gut ecology at species level, the bifidobacterial community harbored by domesticated and CWL animals were investigated using a previously described bifidobacterial ITS-profiling approach (Milani *et al.*, 2014). Remarkably, a PCR amplicon corresponding to the ITS sequences was not obtained for 20 of the 146 fecal samples included in this study, thus indicating absence or very low abundance of bifidobacteria (Table S4, Supporting Information). In order to

exclude that the absence of ITS amplicon for the above-mentioned 20 fecal samples was due to the DNA extraction protocol, a classical cultivation method was employed in order to isolate bifidobacterial species. This cultivation protocol was applied to the 20 fecal samples for which an ITS bifidobacterial PCR amplicon was not achieved as well as to 5 random stools positive to the ITS bifidobacterial PCR. Notably, metagenomics data were confirmed by growth assay on selective media. In this context, no bifidobacterial strains were isolated from the 20 above-mentioned fecal samples, while various bifidobacterial taxa were obtained from the five selected samples that had generated an ITS-based amplicon (Table 4).

Table 4. Isolated bifidobacterial species from fecal samples positive to the ITS bifidobacterial PCR.

Sample	Isolated bifidobacterial species
Canis_lupus_familiaris_10	*Bifidobacterium pseudolongum* *Bifidobacterium longum*
Felis_silvestris_catus_4	*Bifidobacterium breve* *Bifidobacterium pseudolocatenulatum* *Bifidobacterium pseudolongum*
Ammotragus_lervia_1	*Bifidobaterium dentium* *Bifidobacterium longum* *Bifidobacterium pseudolongum*
Equus_ferus_3	*Bifidobacterium animalis* subsp. *animalis* *Bifidobacterium breve* *Bifiodbacterium longum*
Sus_scrofa_2	*Bifidobacterium adolescentis* *Bifidobacterium termophilum*

Therefore, those 20 fecal samples were excluded from further analyses. Bifidobacterial ITS microbial profiling produced a total of 3,470,610 reads that were quality-filtered resulting in a total of 3,117,015 reads with an average of 24,738 filtered reads per sample (Table S4, Supporting Information). The quality filtered sequences were grouped into clusters of identical sequences (OTUs) and then taxonomically classified (Excel File S2, Supporting Information). The obtained ITS data revealed that the domesticated gut was colonized by a higher number of bifidobacterial species with a relative

abundance of >0.01 % compared to the CWL relative group (24 and 18 in domesticated and CWL group, respectively) (Excel File S2, Supporting Information). In addition, even if not statistically significant, the 16S rRNA gene microbial profiling highlighted that the domesticated group showed a higher average relative abundance of the *Bifidobacterium* genus when compared to the CWL mammals (0.49 % ± 0.18 % and 0.22 % ± 0.04 % in the domesticated and CWL groups, respectively). These findings suggest that close contact with humans may have promoted horizontal bacterial transmission leading to subsequent colonization and adaptation of bifidobacterial species to the domesticated animal intestinal tract. Reconstruction of the taxonomic profile of the bifidobacterial species highlighted that only *Bifidobacterium longum* and *Bifidobacterium adolescentis* were present at high prevalence (>80 % and >70 %, respectively) in both analyzed groups (Figure 3). This indicates that these bifidobacterial species have evolved the ability to colonize the gut of a wide range of mammals, as previously suggested by genomics investigations of these species (Duranti *et al.*, 2014, Milani *et al.*, 2014), regardless of domestication (Figure 3). However, the average relative abundance of *B. longum* was statistically higher (Student's t-test p-value <0.05) in the CWL group (21.15 % ±3.36 %) compared to the domesticated group (13.23 % ± 2.22 %). Probably, the acquisition of a higher level of diversity in terms of bifidobacterial species observed in the domesticated group has contributed to the decreased relative abundance of some taxa, including *B. longum*. Interestingly, *Bifidobacterium pseudolongum* showed both a higher average relative abundance (Student's t-test p-value <0.05) and prevalence (>80 %) in the domesticated group as compared to the CWL group (Figure 3), indicating that modulation of diet and lifestyle caused by domestication may have favored colonization and persistence of this taxa. Moreover, *Bifidobacterium animalis* and *Bifidobacterium breve* showed statistically significant increased relative abundance in the domesticated animals as compared to the CWL counterparts (Student's t-test p-value <0.05) (Figure 3). Notably, while *B. animalis* spp. are able to adapt to different niches (Bottacini *et al.*, 2014), *B. breve* species are known to be associated with the human gut (Turroni *et al.*, 2018). Thus, the higher abundance of the latter in

the domesticated animal gut microbiota strengthens the idea that anthropogenic influences and subsequent close contact with humans may have promoted horizontal transmission events.

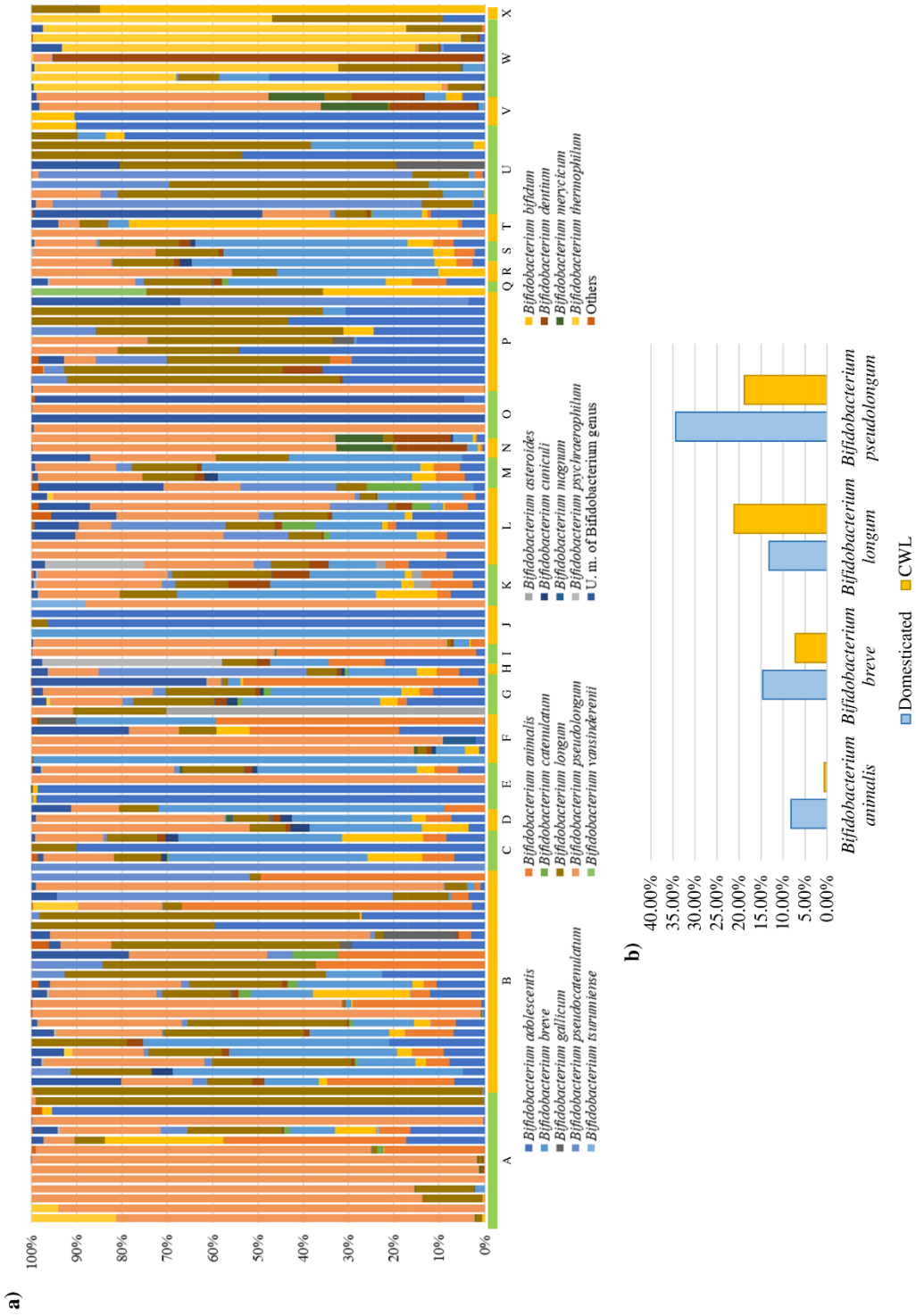

Figure 3. Bifidobacterial profiling of the collected fecal samples. Panel a shows bar plots representing the percentage of the bifidobacterial population of each analyzed sample. Letters at the bottom of the bar plots correspond to the different animal species involved in the study. Sample legend: *Bos Taurus* (A), *Canis lupus familiaris* (B), *Capra hircus* (C), *Equus africanus* (D), *Equus africanus asinus* (E), *Equus ferus caballus* (F), *Felis silvestris catus* (G), *Lama glama* (H), *Mus musculus* (I), *Oryctolagus cuniculus* (J), *Ovis aries* (K), *Sus scrofa domesticus* (L), *Vicugna pacos* (M), *Ammotragus lervia* (N), *Apodemus sylvaticus* (O), *Canis lupus* (P), *Equus africanus* (Q), *Equus ferus* (R), *Felis silvestris* (S), *Lama guanicoe* (T), *Lepus europaeus* (U), *Ovis musimon* (V), *Sus scrofa* (W) and *Vicugna vicugna* (X). Panel b depicts the percentage of those bacterial species whose relative abundance is statistically differs between the domesticated and CWL animals' gut microbiota.

Chapter 6

Deciphering the bifidobacterial populations within the canine and feline gut microbiota

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Characterization of the feline and canine intestinal microbial community by means of 16S rRNA-based microbial profiling

To investigate the taxonomical composition of the intestinal bacterial community of the mammalian species *Canis lupus familiaris* and *Felis catus silvestris*, a total of 161 fecal samples, composed of 23 feline fecal samples and 138 canine stool samples were analysed, representing 7 and 46 distinct feline and canine breeds, respectively, and corresponding to 161 different animals (see Table S1 in the supplemental material). In detail, feline fecal samples were specifically collected for this study and subjected to 16S rRNA gene sequencing, as previously described (Milani *et al.*, 2013). Conversely, the 16S rRNA microbial profiling data of the canine fecal samples were obtained by re-analyzing the 16S rRNA gene sequences from a previous study that followed the same experimental procedures used for feline samples processed here (Alessandri *et al.*, 2019). In this case, as all feline fecal samples belonged to cats that followed a commercial food-based diet, only fecal samples corresponding to the commercial food-fed dogs were taken into consideration to prevent any bias as previously discussed (Alessandri *et al.*, 2019). Illumina sequencing generated a total of 8,732,180 sequenced reads with an average of 53,902 reads per sample. Quality and chimera filtering then generated a total of 8,052,497 filtered sequence reads with an average of 49,706 reads per sample (Table S2). The generated data was used to evaluate bacterial biodiversity through alpha diversity analysis based on the observed exact sequence variants (ESVs). Assessment of the two rarefaction curves corresponding to the average observed for the two mammalian groups, revealed differences in the biodiversity of canine and feline gut microbiota. In detail, Student's t-test statistical analysis (t-value <0.05) revealed a significantly higher level of diversity among the feline fecal bacterial community when compared to that harbored by canine fecal samples (Figure S1a), in line with what had previously been observed (Handl *et al.*, 2011). Additionally, 16S rRNA gene sequencing data were used to reconstruct the canine and feline core gut microbiota, defined as the ensemble of microbial genera that are shared across samples of a defined cohort (Salonen *et al.*, 2012). To identify the core intestinal bacterial community of dogs and cats, only bacterial genera that were identified in at least 80 % of collected

samples and with an average relative abundance of > 0.1 % were considered. Interestingly, while the canine gut microbiota counted 21 bacterial genera fulfilling these two 'core' inclusion criteria, the feline core intestinal community consisted of 33 microbial taxa. In detail, the core gut microbiota of dogs is dominated by *Fusobacterium* (average relative abundance of 24.32 %), *Bacteroides* (13.66 %) and *Prevotella* 9 (a sub-cluster of the *Prevotellaceae* family inferred from available *in silico* data from the SILVA database) (15.18 %), as reported previously (Alessandri *et al.*, 2019). Conversely, in the feline core intestinal population, *Prevotella* 9 was predominant with an average relative abundance of 28.92 % followed by *Bacteroides* (8.32 %), *Romboutsia* (5.28 %) and *Clostridium* sensu stricto 3 (5.32 %). Members of the genus *Fusobacterium*, despite being part of the feline core gut microbiota, displayed a markedly reduced relative abundance (2.34 %) compared to the canine group (Figure S1b and c), indicating that the *Fusobacterium* genus may have undergone specific adaptations in order to colonize the *Canis lupus familiaris* intestine. These taxonomical differences were further substantiated by a hierarchical clustering analysis based on 16S rRNA microbial profiling of the canine and feline fecal samples (Figure 1). Indeed, even though the generated cladogram did not display a clear separation of the collected samples according to these two mammalian groups, most of the feline fecal samples clustered together. Particularly, the cladogram revealed the presence of four main clusters, one of which may be separated into three different subclusters. These findings allowed separation of the collected fecal samples into six distinct gut community state types (GCSTs), each dominated by a minimum of one to a maximum of three bacterial genera with an average relative abundance of >10 %: *Lactobacillus* (GCST 1); *Bacteroides*, *Fusobacterium* and *Faecalibacterium* (GCST 2A); *Fusobacterium* (GCST 2B); *Fusobacterium* and *Bacteroides* (GCST 2C); *Fusobacterium, Prevotella* 9 and *Bacteroides* (GCST 3); and *Prevotella 9* (GCST 4) (Figure 1). Although six sub-clusters were identified, three groups were shown to contain the largest number of samples, i.e., clusters 2C, 3 and 4, which represent 31, 53 and 26 samples, respectively. This suggests that *Fusobacterium*, *Prevotella* 9 and *Bacteroides* are the major players of the canine and feline fecal samples. Clustering of the collected samples according to abundance variation of certain microbial

genera was further corroborated by beta-diversity analysis performed by means of Bray-Curtis distance matrix and represented by principal coordinate analysis (PCoA) (Figure S2). This PCoA clustering analysis was confirmed by a permutational multivariate analysis of variance (PERMANOVA) when the various GCSTs were compared (p-value < 0.05). Interestingly, the latter analysis reveals partial overlap between GCST 2C and 3, probably due to their similar microbial compositions.

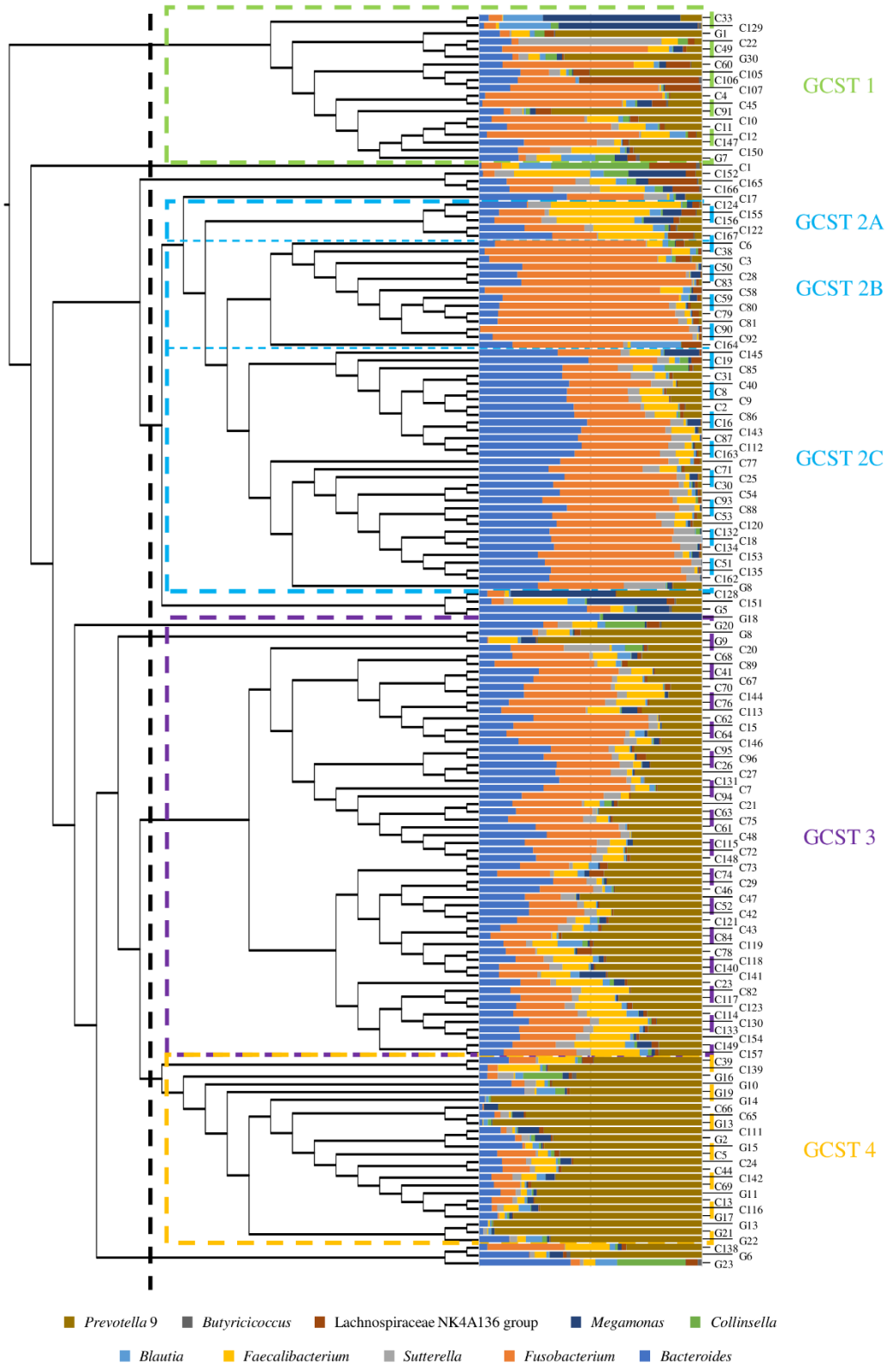

Figure 1. Classification into distinct compositional types of the gut microbiota of dogs and cats. The cladogram on the left displays the hierarchical clustering of the collected samples based on their average 16S rRNA gene microbial profiles. The bar plot in the center depicts the abundance of the 10 most representative microbial taxa of the considered samples. The numbers on the right correspond to the name of collected samples. The colored dotted rectangles separate samples into the six identified Gut Community State Types (GCSTs) whose name is reported on the right with the same GCST color code.

Distribution of bifidobacteria in the intestinal microbial community of dogs and cats

16S rRNA microbial profiling data were exploited to evaluate the relative abundance of the *Bifidobacterium* genus within the canine and feline gut microbiota. Interestingly, while the feline gut microbiota showed an average relative bifidobacterial abundance of 0.46 % with a prevalence of 95.65 %, the canine intestinal microbial community only possessed an average relative bifidobacterial abundance of 0.20 % with a lower prevalence level (74.64 %). Bifidobacterial species have been reported to be universally distributed across the mammalian species, including both cats and dogs, suggesting that these intestinal commensals have evolved a broad host colonization potential (Bunesova *et al.*, 2014, Milani *et al.*, 2017). In this context, the observed lower prevalence level of bifidobacterial species in the gut microbiota of dogs (compared to that found in cats) may be a consequence of the limited sensitivity of the 16S rRNA gene-based microbial profiling (Milani *et al.*, 2014). To overcome this limitation, ITS bifidobacterial profiling was performed by means of genus-specific primers that target the hypervariable ITS region of bifidobacteria, allowing an in-depth phylotype-level community profiling, according to a previously described protocol (Milani *et al.*, 2014). Illumina sequencing of the bifidobacterial ITS region generated a total of 1,771,641 sequenced reads, which when quality- and chimera-filtered resulted in a total of 1,686,399 filtered reads (Table S3). Beta-diversity analysis performed by means of principal coordinate analysis (PCoA) through the Bray-Curtis distance matrix revealed clustering of the datasets based on host species, i.e. *Felis silvestris catus* and *Canis lupus familiaris*, indicating that the gut microbiota of these two domesticated animals each contain a specific bifidobacterial community (Figure S3). Furthermore, the obtained ITS data revealed differences in the number of bifidobacterial species harboured by the intestinal tract of dogs and cats. Indeed, canine fecal samples appear to contain a higher number of known bifidobacterial species with an average relative abundance of ≥ 0.01 % than the feline gut microbiota (50 and 37 bifidobacterial species, respectively). Furthermore, ITS data were used to reconstruct the bifidobacterial core gut microbiota of the two animal companion groups. Specifically,

the bifidobacterial core gut microbiota of cats and dogs were obtained considering only those bifidobacterial species with a prevalence of ≥ 80 %, leading to the identification of 13 core taxa in dogs, of which *Bifidobacterium breve* (average relative abundance 18.11 %), *Bifidobacterium magnum* (13.17 %), *Bifidobacterium longum* subsp. *longum* (11.30 %), *Bifidobacterium adolescentis* (5.82 %), *Bifidobacterium bifidum* (3.86 %), *Bifidobacterium animalis* subsp. *lactis* (3.45 %) coupled with a novel putative bifidobacterial species, i.e. *Bifidobacterium pseudolongum*_new_subsp. (15.28 %) (see below) as the most prevalent and the most abundant bifidobacterial species of the canine gut microbiota (Table S4). Conversely, using the same criteria just seven bifidobacterial species represented the bifidobacterial core gut microbiota of cats, i.e. *B. adolescentis* (24.61 %), *B. breve* (9.18 %), *Bifidobacterium pseudolongum*_new_subsp. (8.18 %) *B. longum* subsp. *longum* (6.87 %), *B. dentium* (4.29 %), *B. animalis* subsp. *lactis* (2.68 %) and *Bifidobacterium crudilactis* (1.17 %). Interestingly, all species that belong to the bifidobacterial core gut microbiota of cats, with the exception of *B. crudilactis*, were also part of the canine bifidobacterial core gut microbiota (Table S4), suggesting that these bifidobacterial species are able to adapt and colonize the intestine of either of these two hosts. Of note, *B. longum* subsp. *longum* and *B. adolescentis* have been detected in the intestinal community of several mammalian species (Milani *et al.*, 2017), while *B. animalis* subsp. *lactis* and *B. dentium* have also been reported to enjoy a cosmopolitan lifestyle (Turroni *et al.*, 2011, Lugli *et al.*, 2019). On the other hand, the high prevalence of bifidobacterial taxa that are typically associated with the human gut, i.e. *B. breve* and *B. catenulatum*, may be the result of a more close or frequent physical contact between a dog and its owner compared to that between cats and humans (Turroni *et al.*, 2018, Alessandri *et al.*, 2019). Indeed, cats generally live a solitary existence and tend to be more linked to their territory rather than to their owners (Driscoll *et al.*, 2009). In this context, the closer relationship between dogs and humans may have promoted colonization of the above-mentioned bifidobacterial species in the canine intestinal tract, presumably through horizontal transfer events. Moreover, statistical analyses were performed in order to evaluate if differences may occur in the bifidobacterial population based on sex, breed or age in the feline and canine gut

microbiota. A hierarchical clustering analysis of the bifidobacterial ITS microbial profiling data revealed that fecal samples did not cluster in separate groups based on animal breed, indicating that this parameter does not impact on the bifidobacterial population (Figure S4). In addition, no significant differences were observed in the bifidobacterial community of feline fecal samples based on sex, whereas two bifidobacterial species, i.e. *Bifidobacterium pseudocatenulatum* and *Bifidobacterium*_new_taxa_9 (see below for the classification of *Bifidobacterium* new taxa), exhibited a significantly higher relative abundance in female canine fecal samples when compared to their male counterparts (p-value <0.05) (Table S5). This suggests that sex may influence the relative abundance of certain minority bifidobacterial species in the canine intestine. Finally, analysis of varianve (ANOVA) was applied to evaluate possible differences in the bifidobacterial composition when samples are grouped based on age, as previously reported, i.e., puppy (0-8 months old), junior (9-24 months old), adult (25-96 months old) and senior (>97 months old) (Alessandri *et al.*, 2019). Interestingly, no significant differences were observed for feline fecal samples, while six bifidobacterial taxa significantly varied in canine fecal samples according to the above-mentioned age groups (p-value < 0.05) (Table S6). However, only two of the latter bifidobacterial species, i.e. *Bifidobacterium*_new_taxa_60 and *Bifidobacterium thermacidophilum* subsp. *porcinum*, exhibited an average relative abundance > 0.05 % in at least one age-group (Table S6). All together these results suggest that the fecal bifidobacterial population of dogs, in contrast to what was observed for cats, may be subject to variations based on age and/or sex.

Investigation of putative novel members of the *Bifidobacterium* genus

Bifidobacterial ITS profiling showed the presence of ESVs with an identity level of <93 % when compared to the currently classified bifidobacterial species, suggesting the presence of putative novel bifidobacterial taxa in the gut microbiota of dogs and cats. In-depth analysis of these putative new taxa was performed by employing a previously described, customized database (Milani *et al.*, 2014, Milani *et al.*, 2017). The latter contains the ITS sequences of publicly available bifidobacterial

(sub)species as well as the ITS sequences corresponding to all other non-bifidobacterial members of the *Bifidobacteriaceae* family in order to avoid misclassification of ITS sequences. This database also included 89 different putative new (sub)species, identified as previously described (Milani *et al.*, 2017). Interestingly, canine and feline fecal samples were characterized by an average relative abundance of 26.19 % and 26.82 % of putative novel bifidobacterial species, respectively, when compared to the overall bifidobacterial community. Remarkably, 67 ESVs were classified as representative of a putative novel taxon of the *Bifidobacterium* genus classified as *Bifidobacterium pseudolongum*_new_subsp. showing a prevalence of >80 % in the gut microbiota of both dogs and cats, suggesting that this taxon has evolved the ability to colonize the intestinal tract of these two mammalian species. Moreover, the canine bifidobacterial-core gut microbiota included two other putative novel species, designated here as *Bifidobacterium*_new_taxa_60 and *Bifidobacterium*_new_taxa_65 (Table S4). Conversely, feline fecal microbiota possessed *Bifidobacterium*_new_taxa_50 and *Bifidobacterium*_new_taxa_55, two putative novel bifidobacterial species that are phylogenetically related to *B. pseudocatenulatum*, with a high average relative abundance (7.59 % and 3.74 %, respectively), but with lower prevalence (65.22 % and 26.09 %, respectively). Isolation and subsequent phenotypical and genomic characterization of these putative novel taxa will be important to decipher their biological relevance in the canine and feline gut.

Covariance of bifidobacterial species among the gut microbiota of dogs and cats

ITS bifidobacterial profiling data sets were then exploited to evaluate the cooccurrence and coexclusion of known and putative novel bifidobacterial species across the fecal samples of dogs and cats by means of Pearson correlation index. Force-driven networks were constructed using the statistically significant (p-value < 0.05, with p values corrected for multiple comparisons) data obtained by considering the bifidobacterial species with an average relative abundance of ≥0.3 % and ≥0.05 % in case of canine and feline fecal samples, respectively. The different relative abundance

cutoffs used to define the co-variance analyses were chosen to have a comparable number of bifidobacterial species for the two mammalian groups.

The force-driven network generated by modularity analysis by means of Gephi Software for bifidobacterial species harbored by the feline gut microbiota highlighted positive interactions among all the considered bifidobacterial species, except for *B. gallinarum*, thus supporting the notion of extensive coevolution among bifidobacterial members of the intestinal microbial community of cats (Figure 2a).

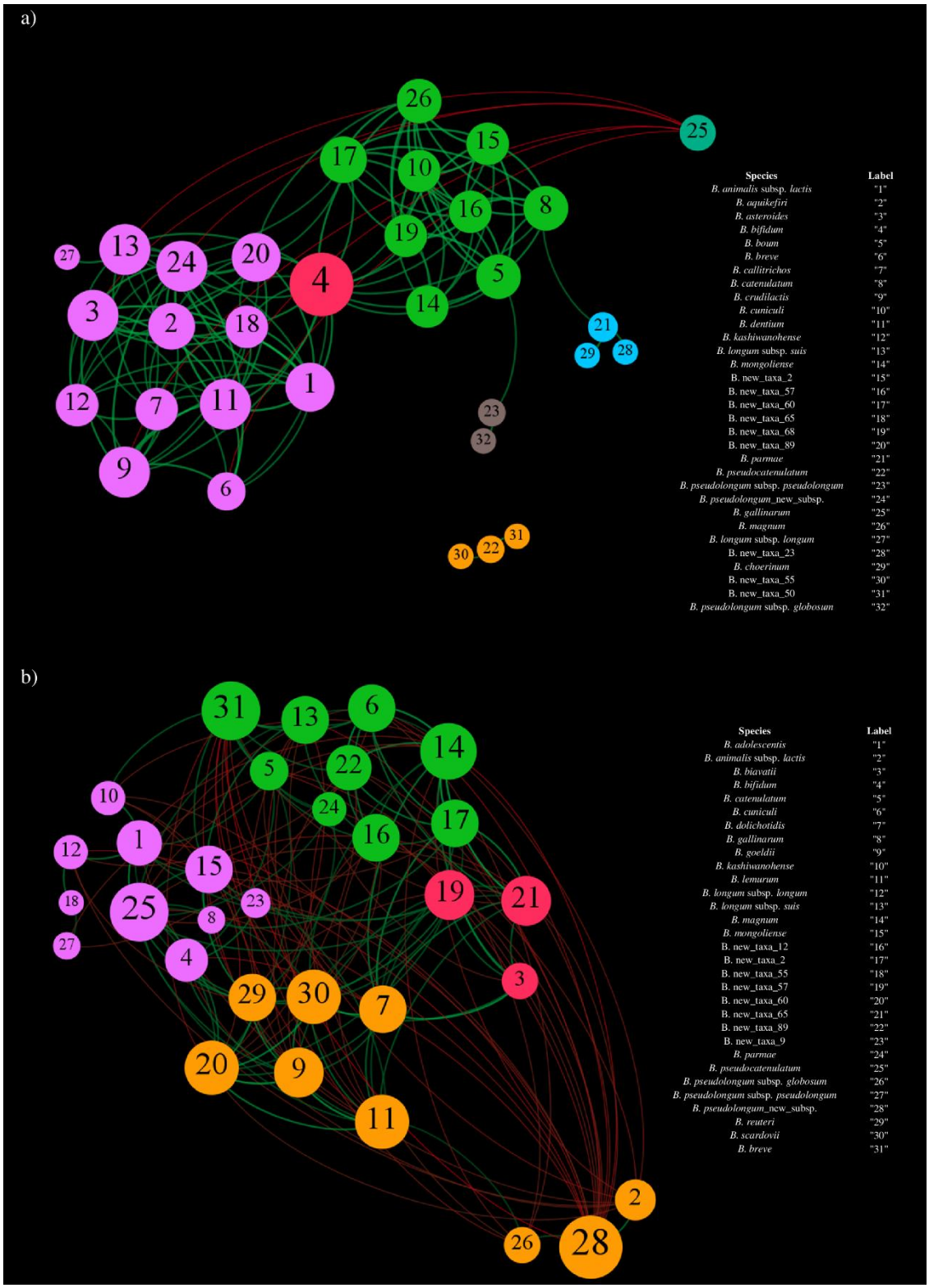

Figure 2: Co-variance network of the most abundant bifidobacterial species of feline and canine fecal microbiota. Panel a depicts the force-driven network obtained by considering the bifidobacterial species with an average relative abundance of ≥0.05 % of the feline fecal samples. Panel b shows the force-driven network constructed by considering the bifidobacterial taxa with an average relative abundance of ≥ 0.3 % of the canine fecal samples. In both cases, the force-driven networks exhibit the predicted co-variances with a p-value of <0.05 between the profiled bifidobacterial species. Moreover, the node size is proportional to the number of co-variances, while the node color indicates the different observed clusters.

Conversely, coexclusion was observed between *B. gallinarum* and six other bifidobacterial taxa, including *Bifidobacterium asteroides*, *B. breve*, *B. crudilactis*, *B. dentium*, *B. longum* subsp. *suis* and *B. longum*_new_subsp. Probably, when present, the high relative abundance of *B. gallinarum* (average of 17.96 %) may play a role in excluding other bifidobacterial species from the colonization of the feline intestinal tract, suggesting a dominant ecological behavior of *B. gallinarum* in the cat's gut microbiota. In this context, despite the fact that *B. gallinarum* was originally isolated from a chicken cecum, its extensive presence in the fecal microbiota of cats may emphasize the cosmopolitan ecological feature of certain bifidobacterial species that may have been prompted by specific predatory/dietary habits, thus corroborating previous reports (Watabe Junko, 1983, Milani *et al.*, 2017). Furthermore, the constructed force-driven covariance network showed the separation of bifidobacterial species into two main clusters and three minor clusters, all characterized by a high degree of cooccurrence among the microbial taxa within a single cluster. Outside this particular case, very few cooccurrence relationships were detected among bifidobacterial species belonging to different clusters. This observation indicates that bifidobacteria may have evolved specific trophic interactions between co-colonizers. Noteworthy, the bifidobacterial species of the two main clusters positively co-varied with *B. bifidum*, suggesting social behavior of this particular bifidobacterial species. The ability of *B. bifidum* to create trophic relationships with other bifidobacterial species was previously observed in humans (Duranti *et al.*, 2019). In this context, *B. bifidum* contains genes encoding glycosyl hydrolases that are predicted to degrade both diet- and host-derived complex glycans, thus allowing the release of partially degraded glycans and subsequent establishment of cross-feeding activities by other bifidobacterial species (Milani *et al.*, 2015). Therefore, as observed in humans, when present, *B. bifidum* also appears to exhibit social behavior within the feline gut microbiota by creating a network of metabolic interactions with other bifidobacterial species.

On the other hand, the force-driven covariance network constructed using profiles of the bifidobacterial species constituting the gut microbiota of dogs, showed the formation of four clusters predicted by modularity analysis by Gephi software, with three unclustered species (Figure 2b). In

this context, while only positive interactions were observed among bifidobacterial species within a single cluster, various coexclusion relationships were identified among bifidobacterial taxa belonging to different clusters. Specifically, *B. pseudolongum*_new_subsp. was shown to display a coexclusion interaction with 17 of the considered bifidobacterial species, while exhibiting just two positive occurrences with *B. animalis* subsp. *lactis* and *B. pseudolongum* subsp. *globosum*. Possibly, as observed here for *B. gallinarum* in the feline gut microbiota, *B. pseudolongum*_new_subsp. has evolved the ability to colonize the canine gut and successfully competes with other bifidobacterial species in this ecological niche.

Bifidobacterial phylogenetic-based clustering of the canine and feline fecal samples

In order to evaluate if a GCST-based clustering of the collected samples is not only observed at genus level, but also at species level, the bifidobacterial ITS profiling data were subjected to hierarchical clustering analysis. The generated cladogram highlighted the division of fecal samples into four main monophyletic clusters named Bifidobacterial Gut Community State Types (BGCSTs) (Figure 3). Furthermore, detailed analysis revealed the presence of three monophyletic subclusters for one of the main BGCSTs. Therefore, when considering bifidobacterial taxa with an average relative abundance of >10 %, the six clusters are dominated by *B. pseudolongum*_new_subsp. (BGCST 1); *B. breve, B. longum* subsp. longum, *B. pseudolongum*_new_subsp. and *B. magnum* (BGCST 2); *B. breve*, *B. longum* subsp. *longum* and *B. magnum* (BGCST 3A); *B. breve, B. longum* subsp. *longum* and *B. pseudolongum*_new_subsp. (BGCST 3B); *B. breve*, *B adolescentis* and *B. pseudolongum*_new_subsp. (BGCST 3C); and *B. adolescentis* (BGCST 4). Interestingly, BGCST 3C and 4 are exclusively represented by feline fecal samples with the exception of two canine fecal samples, while BGCST 1, 2, 3A and 3B encompassed only canine fecal samples (Figure 3).

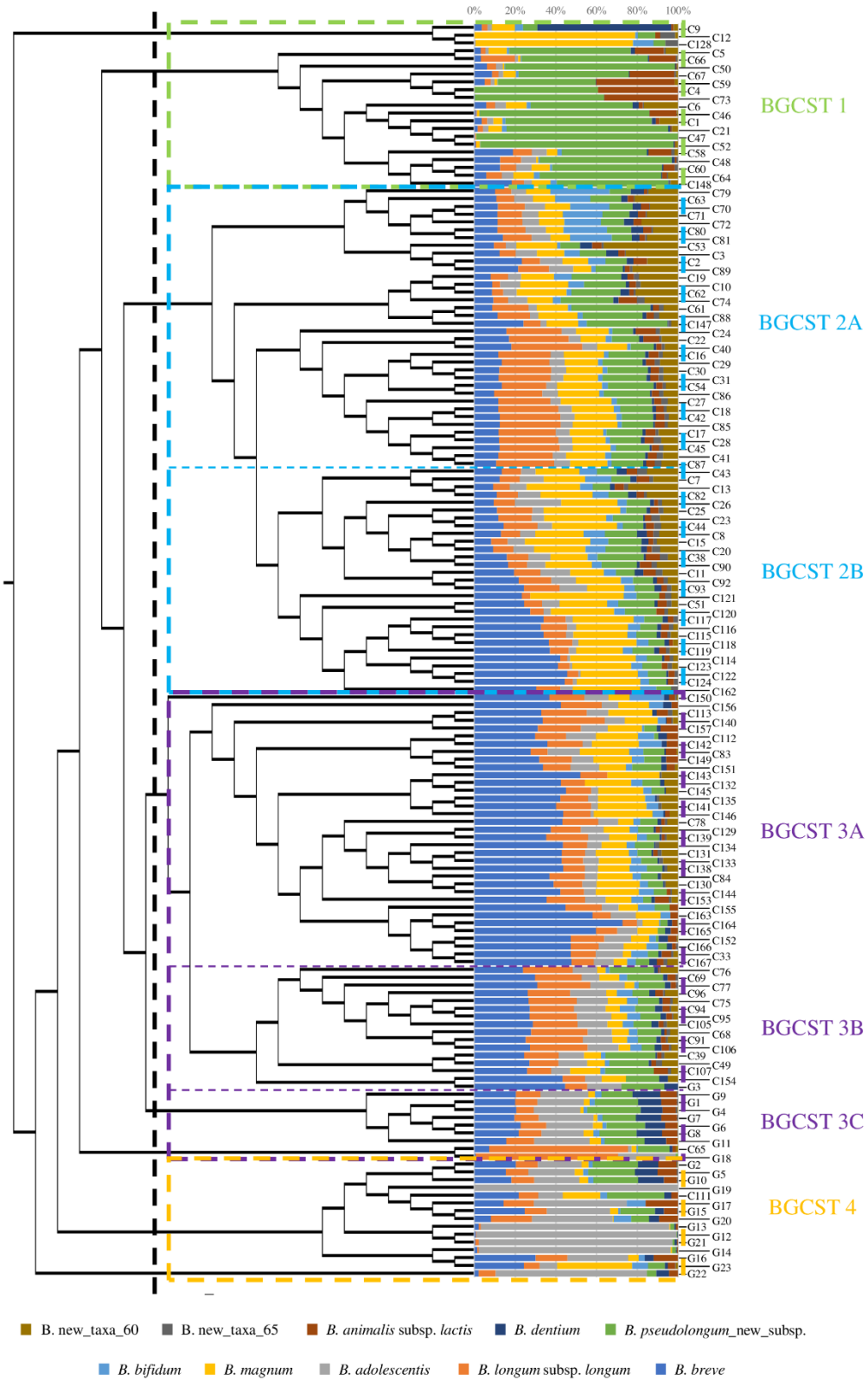

Figure 3: Classification of the canine and feline gut microbiota into distinct types based on their bifidobacterial community. The cladogram on the left depicts the hierarchical clustering of the considered fecal samples based on their average bifidobacterial profiles. The bar plot in the center shows the abundance of the 10 most representative bifidobacterial species of the collected samples, whose name is reported on the right. The colored dotted rectangles divide samples into the eight identified Bifidobacterial Gut Community State Types (BGCSTs), whose designation is reported on the right with the same BGCST color code.

Furthermore, beta-diversity analysis performed by means of Bray-Curtis distance matrix showed a clear separation of the collected fecal samples according to the above-mentioned clusters, a finding that was also confirmed by PERMANOVA (p-value < 0.05) (Figure S5), thus corroborating the presence of recurrent bifidobacterial combinations among the individual canine and feline gut microbiota assemblies. Interestingly, PCoA analysis revealed a partial overlap of samples belonging to the BGCST 2, 3A and 3B subclusters. All together these results suggest that the intestinal communities of dogs and cats harbor distinct bifidobacterial combinations that can be assigned to six different BGCST clusters.

Covariance of bifidobacterial species and other major microbial players of the canine and feline gut microbiota

To investigate the covariance interactions among bifidobacterial species and the other most abundant microbial genera of the canine and feline gut microbiota, a force-driven network was built. Specifically, this cooccurrence analysis was performed involving those bacterial genera with an average relative abundance of >1 % of the gut microbiota of dogs and cats, and by normalizing the bifidobacterial ITS profiling data of each sample for the *Bifidobacterium* genus relative abundance obtained from the 16S rRNA gene sequencing. As revealed by Gephi modularity analysis, bifidobacteria did not cluster together in a single group, but as was observed above, bifidobacterial species established positive interactions among each other, supporting once again the assumption of trophic behavior between various bifidobacterial members of the canine and feline gut microbiota (Figure S6). Conversely, positive interactions were established between certain bifidobacterial species and one of the most abundant microbial genera, i.e. *Prevotella* 9. The latter, in humans, has been associated with a carbohydrate- and fiber-based diet. Indeed, species belonging to the *Prevotella* genus are able to degrade a wide range of plant-derived carbohydrates facilitated by specific glycosyl hydrolase-encoding genes in their genomes (Miyazaki *et al.*, 2005, Flint *et al.*, 2008, De Filippo *et al.*, 2010). In the same manner, bifidobacterial species were reported to have evolved genetic strategies to get access to diet- and host-derived glycans (Milani *et al.*, 2016, Turroni *et al.*, 2018). In

this context, the cooperation observed between bifidobacterial taxa and *Prevotella* 9 may be the result of syntrophic interactions between members of these two saccharolytic microbial genera. Conversely, coexclusion connections were observed between bifidobacterial species and two other main players of the canine and feline gut microbiota, corresponding to *Fusobacterium* and *Bacteroides*. Specifically, the latter are known to be functionally involved in the degradation of protein (Butowski *et al.*, 2019). Indeed, both *Fusobacterium* and *Bacteroides* have been related to a high fat- and protein-based diet (Arumugam *et al.*, 2011, Wu *et al.*, 2011, Schmidt *et al.*, 2018). In this context, the proteolytic features of these microbial taxa may have contributed to the negative correlation with bifidobacterial species known to be saccharolytic microorganisms.

Chapter 7

Multi-omics approaches to decipher the impact of diet and host physiology on the mammalian gut microbiome

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Gut microbiota biodiversity across the mammalian branch of the tree of life

We performed 16S rRNA gene-based microbial profiling of 250 fecal samples corresponding to 77 mammalian species, together forming a broad coverage of the mammalian tree of life (Table S1) (Table S1 in supplemental material). Specifically, the enrolled mammalian species represent 66 omnivores, 63 carnivores, 115 herbivores (encompassing different subclasses according to the physiology of their digestive tracts) and 6 piscivores (Table 1). In this context, because of the difficulties in collecting multiple fecal samples from non-domesticated mammals, some of the fecal samples were collected from wild animals (i.e. wolves or boars) while others were retrieved from animals raised in captivity. Furthermore, difficulties in collecting fecal samples from aquatic mammals significantly restricted the number of piscivore members, being limited to two species of dolphins (three fecal samples per dolphin species) (Table S1). Illumina sequencing produced a total of 15,307,128 reads, with an average of 61,229 reads per sample.

Table 1: List of mammals whose fecal samples were collected for this study, including the number of sampled individuals per mammalian species and their diets.

Common name	Species	Family	Number of sampled individuals	Diet group
African moufflon	*Ammotragus lervia*	Bovidae	2	Herbivore (Polygastric Ruminant)
European bison	*Bison bonasus*	Bovidae	2	
Banteng	*Bos javanicus*	Bovidae	1	
Auroch	*Bos primigenius*	Bovidae	1	
Cow	*Bos taurus*	Bovidae	16	
Goat	*Capra aegagrus hircus*	Bovidae	1	
Goat	*Capra hircus*	Bovidae	4	
Nile lechwe	*Kobus megaceros*	Bovidae	1	
Sheep	*Ovis aries*	Bovidae	4	
Mouflon	*Ovis musimon*	Bovidae	5	
Eland	*Taurotragus oryx*	Bovidae	1	
Deer	*Capreolus capreolus*	Cervidae	1	
Giraffe	*G. camelopardalis*	Giraffidae	2	
Camel	*Camelus bactrianus*	Camelidae	2	Herbivore (Polygastric Tylopoda)
Llama	*Lama glama*	Camelidae	1	
Guanaco	*Lama guanicoe*	Camelidae	3	
Alpaca	*Vicugna pacos*	Camelidae	7	
Vicuna	*Vicugna vicugna*	Camelidae	1	
Pygmy hippopotamus	*Hexaprotodon liberiensis*	Hippopotamidae	5	Herbivore (Polygastric Non-Ruminant 3 Stomach)
Hippopotamus	*Hippopotamus amphibius*	Hippopotamidae	3	
Grey kangaroo	*Macropus giganteus*	Macropodidae	1	
Hare	*Lepus europaeus*	Leporidae	9	Herbivore (Monogastric <100 kg)
European rabbit	*Oryctolagus cuniculus*	Leporidae	4	
European beaver	*Castor fiber*	Castoridae	2	
Patagonian mara	*Dolichotis patagonum*	Caviidae	1	
Capybara	*Hydrochoerus hydrochaeris*	Caviidae	2	
African wild donkey	*Equus africanus*	Equidae	4	Herbivore (Monogastric >100 kg)
Donkey	*Equus africanus asinus*	Equidae	5	
Wild horse	*Equus ferus*	Equidae	3	
Horse	*Equus ferus caballus*	Equidae	10	
Grevy zebra	*Equus grevyi*	Equidae	2	
Zebra	*Equus quagga*	Equidae	2	
Asiatic tapir	*Tapirus indicus*	Tapiridae	1	
Sudamerican tapir	*Tapirus terrestris*	Tapiridae	3	
Asiatic elephant	*Elephas maximus*	Elephantidae	2	
Wolf	*Canis lupus*	Canidae	10	Carnivore
Dog	*Canis lupus familiaris*	Canidae	25	
African wild dog	*Lycaon pictus*	Canidae	1	
Wil cat	*Felis silvestris*	Felidae	2	
Cat	*Felis silvestris catus*	Felidae	4	
European lynx	*Lynx lynx*	Felidae	1	
Lion	*Panthera leo*	Felidae	2	
Asiatic lion	*Panthera leo persica*	Felidae	1	
Jaguar	*Panthera onca*	Felidae	1	
Leopard	*Panthera pardus*	Felidae	1	
Tiger	*Panthera tigris*	Felidae	3	
Meerkat	*Suricata suricatta*	Herpestidae	1	

Fur seal	*Arctocephalus pussilus pussilus*	Otariidae	1	
Sudamerican sea lion	*Otaria flavescens*	Otariidae	1	
Grey seal	*Halichoerus grypus*	Phocidae	2	
Red coati	*Nasua nasua*	Procyonidae	1	
Brown bear	*Ursus arctos*	Ursidae	4	
Armadillo	*Chaetophractus villosus*	Dasypodidae	2	
Hedgehog	*Erinaceus europaeus*	Erinaceidae	1	
Wild boar	*Sus scrofa*	Suidae	8	Omnivore
Pig	*Sus scrofa domesticus*	Suidae	10	
Pygmy marmoset	*Callithrix pygmaea*	Cebidae	1	
Emperor tamarins	*Saguinus imperator*	Cebidae	1	
Cotton-top tamarin	*Saguinus oedipus*	Cebidae	1	
Saimiri	*Saimiri boliviensis peruviensis*	Cebidae	1	
Goeldi tamarin	*Callimico goeldii*	Cebidae	1	
Collared mangbey	*Cercocebus torquatus*	Cercopithecidae	1	
Green cercopithecus	*Chlorocebus pygerythrus*	Cercopithecidae	1	
Red-faced macaque	*Macaca fuscata*	Cercopithecidae	1	
Mandrill	*Mandrillue sphinx*	Cercopithecidae	1	
Human	*Homo Sapiens*	Hominidae	19	
Chimpanzee	*Pan troglodytes*	Hominidae	1	
Bornean orangutan	*Pongo pygmaeus*	Hominidae	1	
Macaque	*Eulemur macaco*	Lemuridae	1	
Lemur	*Lemur catta*	Lemuridae	2	
Red ruffed lemur	*Varecia rubra*	Lemuridae	1	
Black-and-white ruffed lemur	*Varecia variegata*	Lemuridae	1	
Wood mouse	*Apodemus sylvaticus*	Muridae	5	
Mouse	*Mus musculus*	Muridae	2	
Rat	*Rattus rattus*	Muridae	6	
Dolphin	*Delphinus delphis*	Delphinidae	3	Piscivore
Bottlenose dolphin	*Tursiops truncatus*	Delphinidae	3	

Evaluation of the alpha diversity, i.e. the biodiversity of the bacterial population harboured by each sample, was performed through rarefaction curves representing the number of observed operational taxonomic units (OTUs) generated with 100 % identity cutoff and obtained for 10 subsamplings of the total read pool. Average curves obtained for the 28 mammalian taxonomic families included in this study revealed that some herbivorous mammalian species, i.e. *Equidae*, *Camelidae*, *Macropodidae*, *Bovidae*, *Elephantidae* and *Giraffidae* possess a higher gut bacterial biodiversity compared than that of other mammals, supported by Student's t-test *p*-value of <0.001 (Figure 1a).

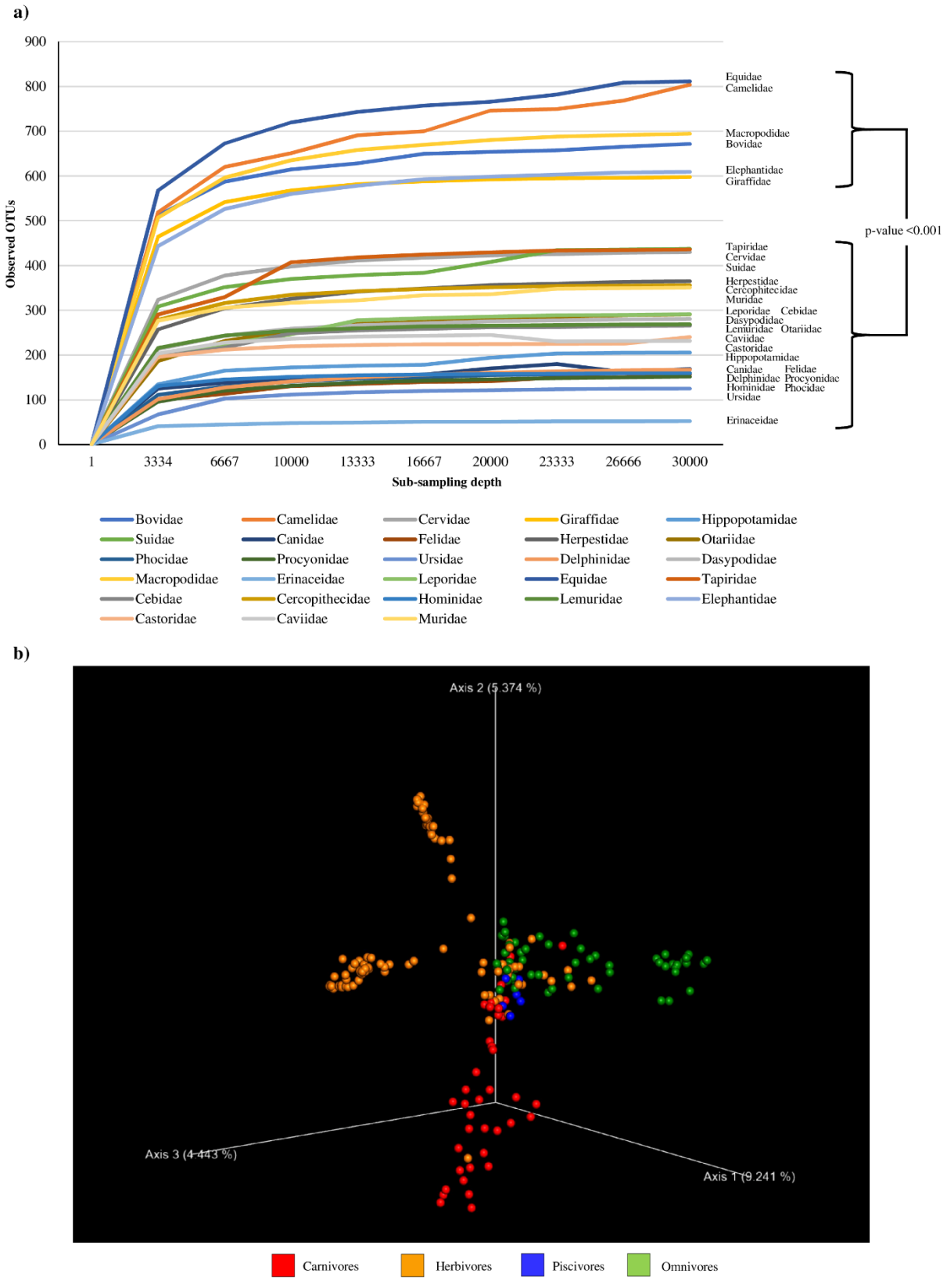


Figure 1. Alpha and Beta diversity of mammals included in this study. Panel a shows the average rarefaction curves obtained for each mammalian taxonomic family through evaluation of the number of observed OTUs up to 30,000 reads. Panel b reports the PCoA representation obtained using the Bray-Curtis index and the genus-level profiles. Samples were colored based on diet, i.e. carnivores, herbivores, piscivores and omnivores.

This observation is confirmed by average diet-based rarefaction curves revealing a significantly higher biodiversity (Student's t-test *p*-value of <0.001) of the gut microbiota of herbivores when compared to that of omnivores or carnivores (the latter including piscivores) (Figure S1a). These data indicate that the overall bacterial biodiversity harboured by the mammalian gut positively correlates with the abundance of plant-based foods in the diet (*p*-value < 0.001), suggestive of a major metabolic role played by bacteria in the gastrointestinal tract of herbivores.

In this context, we also performed a subclassification of the enrolled herbivores based on the physiology of their digestive system (Table S1 and Figure S1b). The average rarefaction curves that we obtained revealed that polygastric herbivores, including ruminants and pseudo-ruminants (Tylopoda), possess a significantly higher gut microbiota biodiversity (Figure S1b), reflecting the key role of foregut bacterial fermentation in herbivores with a multichambered stomach (Dehority, 2002). The only exception was represented by Hippopotamidae, which showed lower biodiversity. Notably, this apparent inconsistency may reflect the peculiar physiology of the three-chambered stomach of these non-ruminant herbivores (Dehority, 2002). In contrast, herbivores with single-chambered stomach showed significant variation in the number of observed OTUs based on their size (Figure S1b). In detail, "lighter" (<100 Kg of average body weight) monogastric herbivores (representing five mammalian species and an associated total of 18 fecal samples) were shown to exhibit lower biodiversity than that of "heavier" (>100 Kg of average body weight) monogastric herbivores (encompassing eight mammalian species and a total of 32 fecal samples). This finding may reflect the fact that small herbivores are cecum fermenters, while heavier herbivores are colon fermenters (Dehority, 2002). For this purpose, cecum fermenters possess an enlarged cecum, which retains small food particles for fermentation while fibrous and less digestible particles pass rapidly through the large intestine. This peculiar physiology of the gastrointestinal tract supports a high-fiber diet without the encumbrance of a large hindgut, thus being advantageous for small animals with a high ratio of food intake to size (Dehority, 2002). In contrast, in colon fermenters the contents of colon and cecum

mix freely and the colon and cecum act as a single fermentation site (Dehority, 2002), possibly supporting the higher bacterial biodiversity observed in heavier monogastric herbivores (Figure S1b).

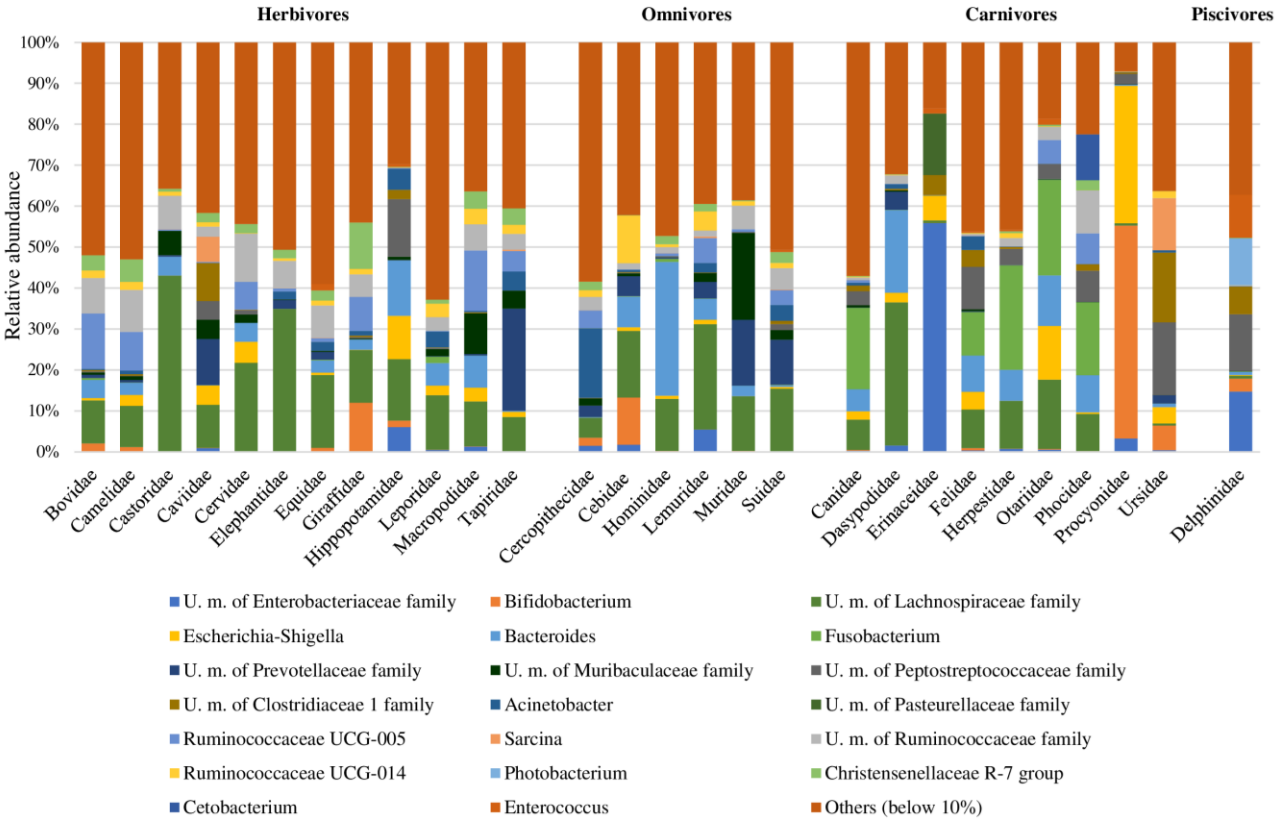
Gut microbiota composition across the mammalian branch of the tree of life

Microbial taxonomic profiles obtained at the genus level were used to perform a beta-diversity analysis using the Bray-Curtis distance matrix and then were represented by means of a principal coordinate analysis (PCoA) plot (Figure 1b and Figure S2a). This analysis revealed clustering of samples based on taxonomic family, as expected, with overlap of families with a similar diet (Figure S2a). In fact, recolouring of the samples based on dietary habits revealed that herbivores, omnivores, and carnivores (including piscivores) clustered separately (Figure 1b), with herbivores forming subclusters, confirming previously published observations (Muegge *et al.*, 2011). In order to detail differences between herbivores, a specific Bray-Curtis PCoA was generated (Figure S2b). The latter revealed three major clusters constituted by i) polygastric ruminants and pseudo-ruminants (Tylopoda), ii) heavier monogastric herbivores, and iii) lighter monogastric herbivores and Hippopotamidae (Figure S2b). These findings highlight that diet, as well as the physiology and anatomy of the herbivorous digestive system, has an impact not only on overall bacterial biodiversity, i.e., number of different bacterial taxa, but also on the gastrointestinal microbiota composition.

Furthermore, in-depth analysis of the microbial taxonomic profiles reconstructed from 16S rRNA gene-based microbial profiling data evidenced similarities between taxonomic families of mammals with an analogous diet (Figure 2a). Details regarding key taxa correlated with specific diets or gastrointestinal physiologies are extensively discussed in the supplemental text. Among the most relevant findings, it is worth mentioning that carnivores and herbivores are characterized by a peculiarly high average abundance of the genus *Fusobacterium* and members of the *Ruminococcaceae* family, respectively (Figure 2b). In this context, it has previously been shown that the *Fusobacterium* genus is generally associated with a protein-rich diet (Vital *et al.*, 2015), while a

high abundance of members of the *Ruminococcaceae* family is related to a fibre-based diet, since the latter are degraders of a wide range of carbohydrates (La Reau & Suen, 2018). Nevertheless, though our findings indicate that members of these two bacterial taxonomic groups play a defining metabolic role for their host, their subgenus phylogeny and genetic potential are still poorly characterized. They therefore represent prime targets for further genomic and functional studies. In this regard, analysis of the herbivorous gut microbiota revealed that the *in silico* predicted genera UCG-005 and UCG-010 of the family *Ruminococcaceae* together represent 18.49 % of the total gut microbial population of polygastric herbivores (Figure S3a and b). Moreover, the small monogastric class (<100 Kg-average body weight) is characterized by a higher number of class-specific taxa than other herbivores (Figure S3b), suggesting that the peculiar gut microbiota composition of cecum fermenters may reflect their shorter transit time and specific energy extraction capabilities when compared to colon fermenters, i.e. heavier monogastric animals (>100 Kg-body average weight), and ruminants (Dehority, 2002).

a)



b)

Phylum	Genus	Carnivores	Herbivores	Omnivores	Piscivores	P-value
Actinobacteria	Collinsella	2.08%	0.03%	0.15%	0.09%	0.000
Actinobacteria	U. m. of Coriobacteriales order	1.10%	0.08%	0.16%	0.05%	0.000
Bacteroidetes	Alloprevotella	2.67%	0.73%	0.63%	0.00%	0.000
Bacteroidetes	Rikenellaceae RC9 gut group	0.20%	3.91%	1.36%	0.02%	0.000
Bacteroidetes	U. m. of Muribaculaceae family	0.44%	1.08%	5.13%	0.05%	0.000
Bacteroidetes	U. m. of Prevotellaceae family	0.43%	2.03%	7.05%	0.02%	0.000
Firmicutes	Blautia	2.50%	0.08%	0.50%	0.07%	0.000
Firmicutes	Enterococcus	0.21%	0.42%	0.17%	10.49%	0.000
Firmicutes	Eubacterium coprostanoligenes group (Ruminococcaceae family)	0.45%	2.53%	1.13%	0.06%	0.000
Firmicutes	Faecalibacterium	1.11%	0.19%	2.65%	0.11%	0.000
Firmicutes	Megamonas	1.27%	0.05%	0.13%	0.00%	0.000
Firmicutes	Ruminococcaceae NK4A214 group	0.19%	1.27%	0.58%	0.05%	0.000
Firmicutes	Ruminococcaceae UCG-005	0.84%	6.47%	2.02%	0.07%	0.000
Firmicutes	Ruminococcaceae UCG-010	0.07%	3.63%	0.18%	0.00%	0.000
Firmicutes	Staphylococcus	0.02%	0.01%	0.03%	4.10%	0.000
Firmicutes	Turicibacter	1.83%	0.15%	0.07%	0.46%	0.000
Firmicutes	U. m. of Clostridiaceae 1 family	2.90%	0.58%	0.36%	6.82%	0.004
Firmicutes	U. m. of Clostridiales order	0.11%	2.46%	0.59%	0.06%	0.000
Firmicutes	U. m. of Clostridiales vadinBB60 group family	0.04%	1.12%	0.49%	0.04%	0.003
Firmicutes	U. m. of Lactobacillales order	0.00%	0.00%	0.00%	2.57%	0.000
Firmicutes	U. m. of Peptostreptococcaceae family	5.93%	1.25%	0.53%	13.84%	0.000
Firmicutes	U. m. of Ruminococcaceae family	1.02%	7.07%	3.44%	0.13%	0.000
Fusobacteria	Fusobacterium	15.37%	0.38%	0.28%	0.10%	0.000
Proteobacteria	Actinobacillus	0.01%	0.00%	0.00%	4.37%	0.000
Proteobacteria	Photobacterium	0.00%	0.00%	0.00%	11.49%	0.000
Proteobacteria	Pseudomonas	2.52%	0.37%	1.99%	9.49%	0.010
Proteobacteria	U. m. of Enterobacteriaceae family	0.40%	0.54%	1.52%	14.73%	0.000
Proteobacteria	Vibrio	0.00%	0.00%	0.00%	1.79%	0.000
Verrucomicrobia	Akkermansia	0.07%	1.34%	0.43%	0.05%	0.000

Figure 2. Impact of diet on mammalian gut microbiota genus-level taxonomic composition. Panel a reports a bar plot of the average genus-level taxonomic composition obtained for each mammalian taxonomic family. Taxonomic families are grouped by diet. "U. m. of" stands for "Unclassified member of". Panel b shows the bacterial genera with average relative abundance being 2X higher in mammals following a specific diet when compared to the other three considered diets. These taxa are highlighted in green.

Covariance of gut colonizers across the mammalian tree of life

The composition and dynamics of the intestinal microbial community rely on an intricate cross-species network of interactions (Coyte & Rakoff-Nahoum, 2019). In this context, previous studies have revealed the existence of both cooperative and competitive behaviours between members of the mammalian gut microbiota (Milani *et al.*, 2017, Garcia-Bayona & Comstock, 2018, Coyte & Rakoff-Nahoum, 2019). In order to investigate such interactions that occur in the gut microbiota across all mammals, we performed a Kendall's tau coefficient covariance analysis using all taxonomic profiles obtained in this study. The data collected were used to construct a force-driven network, where attractive and repulsive forces between nodes correspond to positive and negative covariances with a *P* value of <0.05 between taxa for which a relative abundance of >5 % was observed in at least one sample (Figure 3a). In this context, colouring of the nodes based on modularity class analysis (resolution of 0.6) revealed the presence of three major clusters organised by cooccurring genera, a smaller cluster encompassing just four taxa, and a single microbial genus that does not cluster with any of the other bacterial taxa (Figure 3a). Moreover, node colouring corresponding to taxa found to be associated with specific diets (p-value <0.05) (Figure 2 and 3b) revealed that genera more abundant in herbivores, carnivores and piscivores clustered together, thus suggesting the existence of putative cooperational behaviours between these taxa. In contrast, genera found to be more abundant in omnivores are located near clusters associated with herbivores or carnivores, reflecting the mixed diet followed by omnivorous mammals. This finding may indicate that omnivores are not associated with specific bacterial genera (or *vice versa*), but, rather, possess a combination of bacterial taxa typical of herbivores and carnivores. This notion is in accordance with a previous observation that omnivores do not possess “generalist” bacterial lineages able to digest both plant- and animal-derived compounds but rather a combination of herbivorous and carnivorous specialist bacterial groups (Groussin *et al.*, 2017). In this milieu, it seems that diet may play a major role in modulating the mammalian gut microbiota, resulting in efficient metabolism of dietary food components.

To better detail differences between herbivores and carnivores, the nodes were also colored to report genera showing higher relative abundance (P value <0.05) in either of these two dietary groups (Table S2 and Figure 3c). Since the distance between nodes is weighted on statistically significant cooccurrence and coexclusion interactions, this network analysis revealed that genera found to be more abundant in herbivores form a tighter cluster than carnivore-specific taxa that are spread across the remaining area of the network (Figure 3c). On the basis of this finding, we speculate that bacterial genera involved in the metabolism of plant-derived carbohydrates need a higher level of cooperation to perform complete degradation of such complex carbohydrates, being abundant in the herbivorous diet, into simple sugars. This hypothesis is further supported by the higher average number of covariances observed, as represented by node size, between herbivore-associated genera as compared to those corresponding to carnivores (Figure 3c).

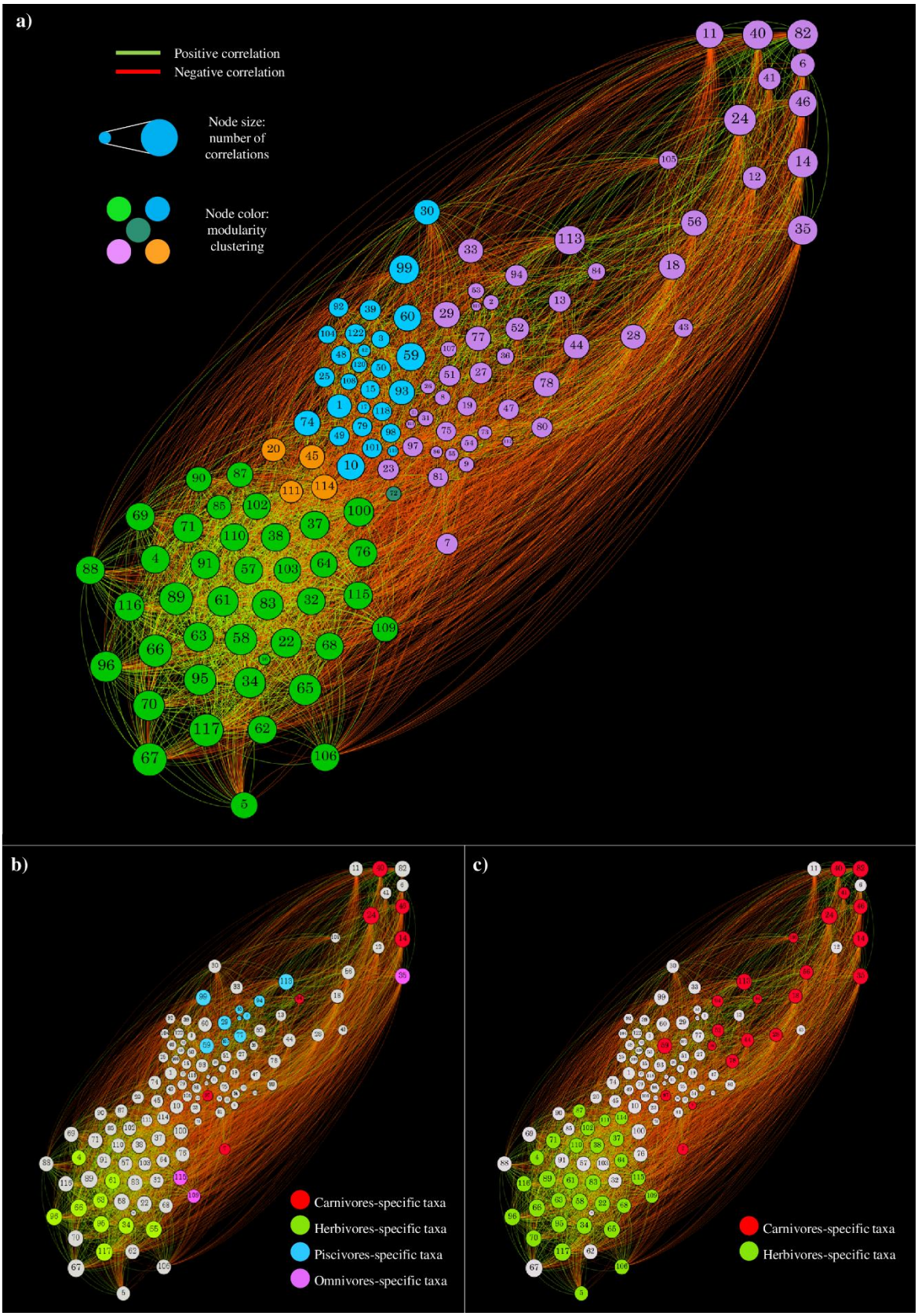


Figure 3. Co-variance force-driven network of genera profiled with relative abundance of >5 % in at least a sample. Nodes represent genera included in the analysis and attraction and repulsion forces are proportional to statistically significant co-variances and co-exclusions obtained using the Kendall's tau correlation coefficient. Node size is proportional to the number of correlations. Panel a reports the network with nodes

colored based on the predicted modularity class (using 0.6 resolution). Panels b and c show the same network with nodes colored to highlight bacterial genera identified as more abundant in a specific diet through analysis of carnivores, herbivores, piscivores and omnivores as well as between only carnivores and herbivores, respectively. Figure numerical legend: *Acinetobacter* (1), *Actinobacillus* (2), *Aeromonas* (3), *Akkermansia* (4), *Alistipes* (5), *Allobaculum* (6), *Alloprevotella* (7), *Anaerococcus* (8), *Asteroleplasma* (9), *Bacillus* (10), *Bacteroides* (11), *Barnesiella* (12) *Bifidobacterium* (13), *Blautia* (14), *Brevundimonas* (15), CAG-352 (16), *Carnobacterium* (17), *Catenibacterium* (18), *Catenisphaera* (19), *Cellulosilyticum* (20), *Cetobacterium* (21), *Christensenellaceae* R-7 group (22), *Clostridium* sensu stricto 1 (23), *Collinsella* (24), *Comamonas* (25), *Corynebacterium* 1 (26), *Cutibacterium* (27), *Dialister* (28), *Enterococcus* (29), *Epulopiscium* (30), *Erysipelotrichaceae* UCG-002 (31), *Erysipelotrichaceae* UCG-004 (32), *Escherichia-Shigella* (33), *Eubacterium coprostanligenes* group (Ruminococcaceae family) (34), *Faecalibacterium* (35), *Faecalibaculum* (36), Family XIII AD3011 group (Clostridiales order) (37), *Fibrobacter* (38), *Flavobacterium* (39), *Fusobacterium* (40), *Helicobacter* (41), *Ignatzschineria* (42), *Lachnospira* (43), *Lactobacillus* (44), *Lysinibacillus* (45), *Megamonas* (46), *Megasphaera* (47), *Myoides* (48), *Paenibacillus* (49), *Pedobacter* (50), *Peptoniphilus* (51), *Peptostreptococcus* (52), *Photobacterium* (53), *Prevotella* 2 (54), *Prevotella* 7 (55), *Prevotella* 9 (56), Prevotellaceae UCG-001 (57), Prevotellaceae UCG-003 (58), Pseudomonas (59), Psychrobacter (60), Rikenellaceae RC9 group (61), *Ruminiclostridium* 6 (62), Ruminococcaceae NK4A214 group (63), Ruminococcaceae UCG-002 (64), Ruminococcaceae UCG-005 (65), Ruminococcaceae UCG-010 (66), Ruminococcaceae UCG-013 (67), Ruminococcaceae UCG-014 (68), Ruminococcaceae V9D2013 group (69), *Ruminococcus* 1 (70), *Saccharofermentans* (71), *Sarcina* (72), *Shuttleworthia* (73), *Solibacillus* (74), *Solobacterium* (75), *Sphaerochaeta* (76), *Staphylococcus* (77), *Streptococcus* (78), *Streptomyces* (79), *Subdoligranulum* (80), *Succinivibrio* (81) *Sutterella* (82), *Treponema* 2 (83), *Turicibacter* (84), U. m. of Ricketsiales order (85), U. m. of WPS-2 phylum (86), U. m. of Bacteroidales BS11 gut group family (87), U. m. of Bacteroidales order (88), U. m. of Bacteroidales RF16 group family (89), U. m. of Bacteroidales UCG-001 family (90), U. m. of Bacteroidia class (91), U. m. of Burkholderiaceae family (92), U. m. of Caulobacteriaceae family (93), U. m. of Clostridiaceae 1 family (94), U. m. of Clostridiales vadinBB60 group family (96), U. m. of Coriobacteriales order (97), U. m. of Cyanobacteria phylum (98), U. m. of Enterobacteriaceae family (99), U. m. of Erysipelotrichaceae family (100), U. m. of Eukaryota kingdom (101), U. m. of F082 family (102), U. m. of Firmicutes phylum (103), U. m. of Flavobacteriaceae family (104), U. m. of Gammaproteobacteria class (105), U. m. of Lachnospiraceae family (106), U. m. of Lactobacillales order (107), U. m. of Moraxellaceae family (108), U. m. of Muribaculaceae family (109), U. m. of p-251-o5 family (110), U. m. of p-2534-18B5 gut group family (111), U. m. of Pasteurellaceae family (112), U. m. of Peptostreptooccaceae family (113), U. m. of Planococcaceae family (114), U. m. of Prevotellaceae family (115), U. m. of Rhodospirillales order (116), U. m. of Ruminococcaceae family (117), U. m. of Sphingomonadaceae family (118), U. m. of Verrucomicrobiae class (119), U. m. of Weekellaceae family (120), *Vibrio* (121), *Vitreoscilla* (122) and *Yersinia* (123).

Functional characterization of the mammalian gut microbiota

The 16S rRNA gene-based microbial profiling analysis revealed substantial differences in the taxonomic composition of the 250 collected fecal samples based on diet and physiology of the digestive system. For this reason, in order to trace potential differences in the functional repertoire of mammalian gut microbial populations, a shotgun metagenomic approach was performed for 24 fecal samples. Specifically, to obtain a balanced analysis, fecal samples were chosen in order to be equally divided per diet category, with exclusion of omnivores due to their extreme complex and variable diet (Table S3). Furthermore, animals included in the same group were chosen randomly to cover multiple sampling sites in order to limit geographical biases. Data retrieved from shotgun sequencing comprised a total of 221,797,722 reads that were subjected to quality filtering and removal of host-related sequences based on publicly available genomes of the sampled animals, resulting in a total of 205,386,184 reads with an average of 8,557,758 reads per sample (Table S3). The obtained sequence datasets were then subjected to metabolic pathway prediction based on the MetaCyc database. Shotgun metagenomics data revealed that the gut microbiota of piscivores encode the highest number of pathways (constituting an average of >0.001 % reads of the datasets) and a higher number of pathways with lower abundance compared to both other diets (Figure 4), thus allowing to formulate the hypothesis that aquatic life and correlated diet induced extensive shift in the metabolic potential of the gut microbiota of these piscivores (further details related to data collected from piscivores [dolphins] and their relative functional assessment are reported in the supplemental text). Furthermore, statistical analysis revealed that carnivores possess a lower number of pathways with differential (higher or lower) abundance than animals on other diets (Bonferroni post-hoc test P value <0.05) (Figure 4). In-depth evaluation of degradative pathways showing higher abundance for a specific diet (Bonferroni post-hoc test P value <0.05) (Figure 4; Table S5) revealed, as expected, that the herbivore gut microbiome is enriched in carbohydrate degradation pathways in comparison to that of carnivores and piscivores (Table S4). Particularly, most of the predicted pathways were related to

the breakdown of typical plant carbohydrates, i.e. xylose, arabinose, sucrose, starch and maltose (Jones *et al.*, 2008, Milani *et al.*, 2015, Kotake *et al.*, 2016) (Table S5), predicting that the gut microbiome has a greater capacity to recover energy from a plant/vegetable-based diet. In contrast, the carnivore gut microbiome is characterized by a higher number of pathways related to choline degradation coupled with the super-pathway of trimethylamine degradation (Table S5). Notably, choline, a quaternary amine principally found in meats, is known as precursor of trimethylamine (Rath *et al.*, 2017, Wang *et al.*, 2019). In this context, the microbial intestinal community associated with carnivores seems to have developed activities capable of degrading meat components and its derived by-products, thus strengthening previous observations which suggested that the carnivore microbiome is specialized to derive energy from protein degradation (Muegge *et al.*, 2011). Collectively these findings support the notion that diet plays a role in modulating the taxonomic composition of the intestinal microbial community, with a consequent impact of the metabolic pathways encoded by these mammalian intestinal microbial communities.

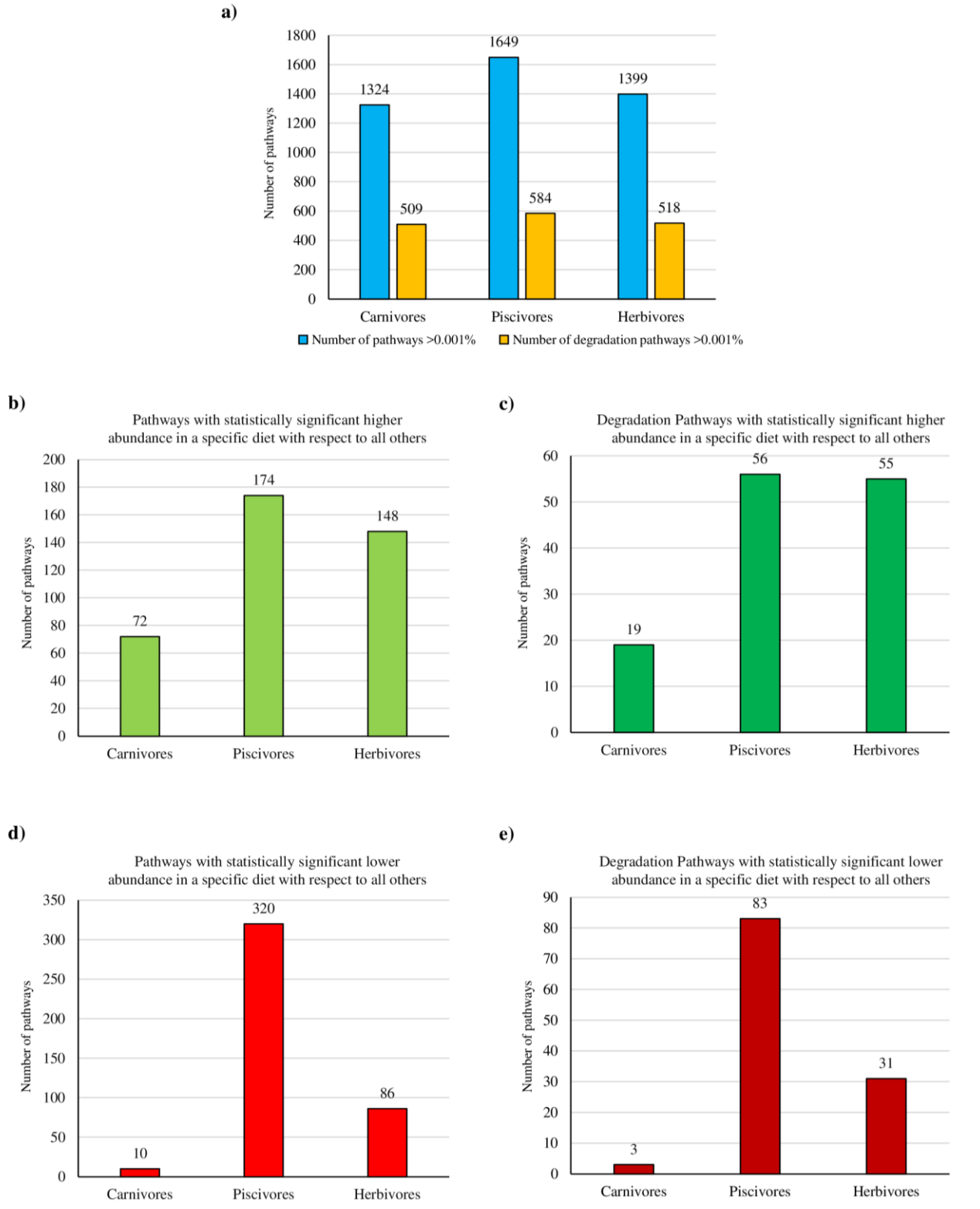


Figure 4. Metabolic pathways prediction in Carnivores, Piscivores and Herbivores. Panel a shows the number of pathways detected with abundance >0.001 %. Panels b and c report the sum of the number of all pathways and degradative pathways, respectively, that showed a significantly higher abundance in a specific diet when compared to the other two considered diets observed through the application of an ANOVA post-hoc Bonferroni statistical analysis. Panels d and e display the sum of the number of all pathways and degradative pathways, respectively, with significantly lower abundance in a specific diet when compared to the other two.

Differences between the gut glycobiomes of carnivores and herbivores

Shotgun metagenomic data were also used to reconstruct the glycobiome, i.e. the genetic repertoire responsible for breakdown of complex carbohydrates. Details of the variations in the gut microbiota glycobiome based on diet (herbivore, carnivore and piscivore) are reported in the supplemental text. Focusing on the comparison between the glycobiome profiles of carnivores and herbivores, we performed a statistical analysis using Student's t-test. Results revealed that a large number of glycosyl hydrolase (GH) families possess differential abundance between the representatives of the two considered diets (Table 2; Table S6). In this context, a marked commitment of carnivores toward the degradation of animal-derived host glycans and their degradation products (GH20, GH33, GH92, GH101, GH123, GH125 and GH129) was noted, as well as α(1→4) linked glucose polysaccharides (GH15, GH63 and GH126) such as the animal storage carbohydrate glycogen (Table S6). Moreover, carnivores showed higher abundance of GH families involved in the degradation of chitin, chitosan and chitobiose (GH19, GH23, GH84, GH85), probably due to the ingestion of chitinous structures (Table S6). In contrast, herbivore data extended the above-observed specialization of their microbiota toward the metabolism of plant-related polysaccharides such as cellulose, xylans and galactans (GH9, GH10, GH11, GH12, GH16, GH26, GH31, GH39, GH42, GH43, GH44, GH51, GH53, GH67, GH74 and GH120) and also highlighted a commitment toward the degradation of fungal polysaccharides such as mycodextran (GH87) (Table S6).

Dissection and statistical analysis of glycobiome data revealed that the gut microbiomes of carnivores, piscivores and herbivores encode a specific repertoire of enzymes to allow energy extraction from dietary carbohydrates, suggesting that the bacterial populations harbored by the mammalian gut exert specific metabolic roles that are associated with the particular diet of their host.

Table 2: List of GH families with statistically significant higher or lower abundance based on diet.

GH family	Carnivores	Piscivores	Herbivores
GH2	9.11%	**1.98%**	8.01%
GH3	4.65%	**1.35%**	5.47%
GH9	0.10%	0.25%	**0.68%**
GH10	0.18%	0.10%	**0.57%**
GH17	0.02%	**0.59%**	0.00%
GH19	0.04%	**0.30%**	0.01%
GH20	**2.54%**	1.04%	1.46%
GH23	1.95%	**13.36%**	1.04%
GH24	**0.23%**	0.05%	0.11%
GH26	0.16%	0.00%	**0.30%**
GH27	0.25%	0.05%	**0.57%**
GH29	1.52%	**0.57%**	1.43%
GH31	1.97%	**0.80%**	**2.57%**
GH33	0.79%	0.62%	**0.30%**
GH35	0.57%	**0.09%**	0.53%
GH39	0.01%	0.00%	**0.15%**
GH43	2.48%	0.48%	**4.14%**
GH51	0.60%	0.30%	**1.69%**
GH53	0.05%	0.08%	**0.31%**
GH67	0.09%	0.00%	**0.29%**
GH74	0.00%	0.00%	**0.08%**
GH100	**0.01%**	0.00%	0.00%
GH102	0.05%	**0.29%**	0.03%
GH103	0.05%	**0.42%**	0.03%
GH110	0.16%	**5.66%**	0.10%
GH129	**0.05%**	0.00%	0.01%
GH130	0.54%	**0.00%**	0.55%

*percentages in bold indicate Bonferroni post-hoc test p-value <0.05 when compared to other groups.

Metatranscriptomic analysis of the microbiomes of carnivores and herbivores

Metagenomics data provided interesting information regarding the functional commitment of the gut microbiota of herbivores and carnivores towards metabolism of specific dietary components. In order to evaluate whether transcriptional profiles of these microbiomes reflect such observations, we performed metatranscriptome analysis of fecal samples from four carnivores and four herbivores (Table S7), which were selected in order to represent various animal genera. Sequenced metatranscriptome data sets were processed for removal of host DNA through mapping against a custom database of host genomes resulting in a total of 38,921,420 reads with an average of 4,865,177

reads per sample, and the latter were further subjected to prediction of the expressed glycobiome and repertoire of degradation pathways (Table S5 and S6; Figure 5).

Inspection of transcriptional data revealed that the range of pathways involved in the breakdown of typical plant carbohydrates, i.e. xylose, arabinose and starch, found to be more abundant in herbivores based on shotgun metagenomic data (Table S5), are also more expressed in animals following this diet (Table S5). Similarly, analysis of the expressed glycobiomes focusing on GH families showing differential abundance in metagenomic data evidenced that genetic members of the GH9, GH26, GH39, GH43, GH51, GH67 and GH74 glycosyl hydrolase families, predicted to be involved in the breakdown of plant-related carbohydrates, are more expressed in herbivores. In contrast, genes encoding GH20, GH33 and GH129 family enzymes, which are predicted to be involved in degradation of host-derived glycans, showed higher transcription levels in carnivores (Table S6). Notably, these data further strengthen the assumption of an extensive specialization of the gut microbiota of mammals in facilitating the metabolism of specific dietary compounds in terms of the encoded genetic repertoire and being corroborated by their transcription patterns.

a)

	Canis_lupus_familiaris_1	Felis_silvestris_catus_1	Lynx_lynx_1	Panthera_tigris_1	Capra_hircus_1	Lama_glama_1	Dolichotis_patagonum_1	Equus_ferus_caballus_1	Average abundance in Carnivores	Average abundance in Herbivores	Student's t-test p-value
GH5	0.0244%	0.0201%	0.2997%	0.0314%	0.9828%	0.5239%	2.1412%	1.9077%	0.0939%	1.3889%	0.015
GH9	0.0000%	0.0000%	0.0000%	0.0000%	1.3293%	0.4136%	1.7674%	1.0648%	0.0000%	1.1438%	0.007
GH27	0.2033%	0.0000%	0.0053%	0.0000%	0.3843%	0.1517%	0.1338%	0.3549%	0.0521%	0.2562%	0.049
GH29	0.1139%	0.0402%	0.0210%	0.0000%	0.6363%	0.3447%	0.1246%	0.4880%	0.0438%	0.3984%	0.019
GH30	0.0000%	0.0000%	0.0000%	0.0000%	0.3906%	0.1448%	0.7245%	0.3549%	0.0000%	0.4037%	0.015
GH39	0.0000%	0.0000%	0.0053%	0.0000%	0.0504%	0.1310%	0.4845%	0.4880%	0.0013%	0.2885%	0.047
GH51	0.0163%	0.0402%	0.0158%	0.0026%	0.2898%	0.3516%	0.5999%	1.1535%	0.0187%	0.5987%	0.026
GH66	0.0000%	0.0000%	0.0000%	0.0000%	0.0630%	0.0758%	0.1061%	0.0887%	0.0000%	0.0834%	0.000
GH94	0.1383%	0.0201%	0.2944%	0.0617%	0.8190%	1.3304%	1.2460%	1.2866%	0.1286%	1.1705%	0.000
GH97	0.0000%	0.0000%	0.0000%	0.0000%	0.3969%	0.2550%	0.1200%	0.5768%	0.0000%	0.3372%	0.014
GH105	0.0163%	0.0000%	0.0000%	0.0000%	0.0756%	0.1792%	0.1061%	0.1331%	0.0041%	0.1235%	0.002
GH112	0.0244%	0.0803%	0.0210%	0.0000%	0.1134%	0.0827%	0.0508%	0.1331%	0.0314%	0.0950%	0.043
GH120	0.0000%	0.0402%	0.0000%	0.0000%	0.2268%	0.2275%	0.0923%	0.3993%	0.0100%	0.2365%	0.012
GH130	0.0000%	0.0000%	0.0000%	0.0000%	0.2079%	0.1930%	0.2630%	0.4437%	0.0000%	0.2769%	0.003

b)

	Canis_lupus_familiaris_1	Felis_silvestris_catus_1	Lynx_lynx_1	Panthera_tigris_1	Capra_hircus_1	Lama_glama_1	Dolichotis_patagonum_1	Equus_ferus_caballus_1	Average abundance in Carnivores	Average abundance in Herbivores	Student's t-test p-value
L-citrulline degradation	0.0083%	0.0236%	0.0193%	0.0251%	0.0676%	0.0586%	0.0626%	0.0684%	0.0190%	0.0643%	0.000
naphthalene degradation (aerobic)	0.0011%	0.0000%	0.0002%	0.0000%	0.0133%	0.0103%	0.0071%	0.0080%	0.0003%	0.0097%	0.000
superpathway of beta-D-glucuronide and D-glucuronate degradation	0.0122%	0.0250%	0.0010%	0.0200%	0.0712%	0.0514%	0.0898%	0.0858%	0.0146%	0.0746%	0.000
L-ornithine degradation I (L-proline biosynthesis)	0.0057%	0.0215%	0.0145%	0.0075%	0.0423%	0.0324%	0.0516%	0.0576%	0.0123%	0.0460%	0.000
myo-, chiro- and scyllo-inositol degradation	0.0104%	0.0139%	0.0129%	0.0225%	0.0491%	0.0499%	0.0640%	0.0885%	0.0150%	0.0629%	0.000
D-fructuronate degradation	0.0078%	0.0056%	0.0010%	0.0200%	0.0496%	0.0275%	0.0513%	0.0402%	0.0086%	0.0422%	0.000
K-carrageenan degradation	0.0124%	0.0000%	0.0002%	0.0000%	0.0384%	0.0415%	0.0182%	0.0322%	0.0032%	0.0325%	0.000
superpathway of hexuronide and hexuronate degradation	0.0133%	0.0444%	0.0011%	0.0200%	0.0814%	0.0582%	0.1011%	0.0885%	0.0197%	0.0823%	0.000
superpathway of nicotinate degradation	0.0289%	0.0333%	0.0231%	0.0251%	0.0675%	0.0600%	0.0700%	0.1059%	0.0276%	0.0758%	0.000
L-glutamate degradation VI (to pyruvate)	0.0217%	0.0312%	0.0011%	0.0200%	0.0624%	0.0616%	0.0569%	0.0389%	0.0185%	0.0549%	0.000
nicotinate degradation III	0.0289%	0.0333%	0.0195%	0.0251%	0.0616%	0.0597%	0.0691%	0.1046%	0.0267%	0.0737%	0.001
phenylacetate degradation I (aerobic)	0.0067%	0.0076%	0.0116%	0.0125%	0.0335%	0.0571%	0.0251%	0.0416%	0.0096%	0.0393%	0.001
carbon tetrachloride degradation II	0.0478%	0.0278%	0.0094%	0.0401%	0.2152%	0.2586%	0.1078%	0.1354%	0.0313%	0.1793%	0.001
myo-inositol degradation I	0.0085%	0.0139%	0.0100%	0.0225%	0.0424%	0.0434%	0.0622%	0.0871%	0.0137%	0.0588%	0.001
L-glutamine degradation I	0.8989%	0.4387%	1.0230%	0.7917%	0.1837%	0.2923%	0.2567%	0.3888%	0.7881%	0.2804%	0.001
D-galactonate degradation	0.0033%	0.0028%	0.0000%	0.0000%	0.0271%	0.0082%	0.0297%	0.0161%	0.0015%	0.0203%	0.001
superpathway of microbial D-galacturonate and D-glucuronate degradation	0.0189%	0.0694%	0.0590%	0.0376%	0.1114%	0.0776%	0.1186%	0.1488%	0.0462%	0.1141%	0.001
L-tryptophan degradation II (via pyruvate)	0.0000%	0.0000%	0.0000%	0.0000%	0.0184%	0.0065%	0.0060%	0.0107%	0.0000%	0.0104%	0.001
L-rhamnose degradation I	0.0063%	0.0042%	0.0007%	0.0025%	0.0200%	0.0141%	0.0154%	0.0349%	0.0034%	0.0211%	0.001
beta-D-glucuronide and D-glucuronate degradation	0.0043%	0.0194%	0.0000%	0.0000%	0.0216%	0.0239%	0.0385%	0.0456%	0.0060%	0.0324%	0.001
melibiose degradation	0.0007%	0.0014%	0.0000%	0.0000%	0.0063%	0.0071%	0.0021%	0.0107%	0.0005%	0.0066%	0.001
heme degradation II	0.0070%	0.0118%	0.0034%	0.0025%	0.0460%	0.0171%	0.0442%	0.0778%	0.0062%	0.0463%	0.001
oxalate degradation I / 2-oxobutanoate degradation II	0.0070%	0.0118%	0.0034%	0.0025%	0.0460%	0.0166%	0.0442%	0.0778%	0.0062%	0.0461%	0.002
L-arabinose degradation IV / xylose degradation IV	0.0461%	0.0333%	0.0069%	0.0100%	0.1596%	0.1798%	0.0424%	0.1153%	0.0241%	0.1243%	0.002
benzoyl-CoA degradation II (anaerobic)	0.0209%	0.0292%	0.0034%	0.0075%	0.0618%	0.0442%	0.0523%	0.1140%	0.0152%	0.0680%	0.002
chlorophyll a degradation II	0.0070%	0.0118%	0.0044%	0.0025%	0.0460%	0.0166%	0.0442%	0.0778%	0.0064%	0.0461%	0.002
nucleoside and nucleotide degradation (archaea)	0.0435%	0.0403%	0.0050%	0.0877%	0.0813%	0.2100%	0.1653%	0.1233%	0.0441%	0.1450%	0.002
guanosine nucleotides degradation III	0.0152%	0.0326%	0.0188%	0.0175%	0.0475%	0.0317%	0.0599%	0.0362%	0.0210%	0.0438%	0.002
naphthalene degradation to acetyl-CoA	0.0011%	0.0000%	0.0003%	0.0000%	0.0133%	0.0103%	0.0071%	0.0013%	0.0003%	0.0080%	0.002
4-deoxy-L-threo-hex-4-enopyranuronate degradation	0.0083%	0.0035%	0.0134%	0.0276%	0.0402%	0.0188%	0.0442%	0.0389%	0.0132%	0.0355%	0.002
choline degradation II	0.0054%	0.0076%	0.0005%	0.0050%	0.0007%	0.0002%	0.0002%	0.0000%	0.0046%	0.0003%	0.003
5-hydroxymethylfurfural degradation	0.0000%	0.0000%	0.0000%	0.0000%	0.0244%	0.0142%	0.0145%	0.0013%	0.0000%	0.0136%	0.003
furfural degradation	0.0000%	0.0000%	0.0000%	0.0000%	0.0244%	0.0142%	0.0145%	0.0013%	0.0000%	0.0136%	0.003
oxalate degradation V	0.0000%	0.0000%	0.0001%	0.0000%	0.0008%	0.0005%	0.0002%	0.0013%	0.0000%	0.0007%	0.003
D-glucarate degradation II	0.0000%	0.0000%	0.0000%	0.0000%	0.0003%	0.0005%	0.0005%	0.0000%	0.0000%	0.0003%	0.003
superpathway of glucose and xylose degradation	0.1024%	0.0958%	0.0044%	0.0952%	0.2760%	0.1652%	0.6399%	0.4022%	0.0744%	0.3708%	0.003
quinate degradation II	0.0000%	0.0000%	0.0020%	0.0000%	0.0099%	0.0019%	0.0064%	0.0040%	0.0005%	0.0056%	0.004
chlorophyll a degradation I	0.0070%	0.0118%	0.0182%	0.0025%	0.0471%	0.0166%	0.0449%	0.0791%	0.0099%	0.0469%	0.004
D-sorbitol degradation II	0.0000%	0.0000%	0.0000%	0.0000%	0.0029%	0.0017%	0.0002%	0.0013%	0.0000%	0.0016%	0.004
3,6-anhydro-alpha-L-galactopyranose degradation	0.0046%	0.0028%	0.0000%	0.0125%	0.0286%	0.0130%	0.0338%	0.0121%	0.0050%	0.0219%	0.004
sulfolactate degradation I	0.0017%	0.0000%	0.0003%	0.0000%	0.0130%	0.0163%	0.0030%	0.0295%	0.0005%	0.0154%	0.004
pectin degradation II	0.0000%	0.0000%	0.0000%	0.0000%	0.0008%	0.0006%	0.0035%	0.0027%	0.0000%	0.0019%	0.004
L-tryptophan degradation I (via anthranilate)	0.0000%	0.0000%	0.0000%	0.0000%	0.0060%	0.0074%	0.0016%	0.0013%	0.0000%	0.0041%	0.005
starch degradation III	0.0000%	0.0000%	0.0000%	0.0000%	0.0041%	0.0000%	0.0069%	0.0080%	0.0000%	0.0048%	0.005
L-asparagine degradation I	0.0065%	0.0007%	0.0044%	0.0050%	0.0322%	0.0065%	0.0187%	0.0161%	0.0042%	0.0184%	0.005
D-galacturonate degradation I	0.0087%	0.0354%	0.0001%	0.0125%	0.0489%	0.0310%	0.0762%	0.0335%	0.0142%	0.0474%	0.005
autoinducer AI-2 degradation	0.0000%	0.0000%	0.0000%	0.0000%	0.0018%	0.0019%	0.0009%	0.0000%	0.0000%	0.0012%	0.005
L-lyxose degradation	0.0063%	0.0000%	0.0001%	0.0025%	0.0070%	0.0082%	0.0092%	0.0214%	0.0022%	0.0115%	0.005
superpathway of dimethylsulfoniopropanoate degradation	0.0100%	0.0090%	0.0008%	0.0000%	0.0067%	0.0157%	0.0163%	0.0161%	0.0050%	0.0137%	0.005
2-aminoethylphosphonate degradation I	0.0000%	0.0014%	0.0001%	0.0000%	0.0065%	0.0052%	0.0016%	0.0013%	0.0004%	0.0037%	0.005
superpathway of methanogenesis	0.1128%	0.3096%	0.0143%	0.3783%	0.9144%	0.9832%	0.4444%	0.4612%	0.2038%	0.7008%	0.028

Figure 5. Metatranscriptome profiles of carnivores and herbivores. Panel a shows the transcriptional abundance (as a proportion of the total glycobiome) of GH genes with statistically different abundance in carnivores and herbivores. GHs in red show similar abundance in the metagenomes of carnivores and herbivores. Panel b reports the transcriptional abundance (as a proportion of all predicted metabolic pathways) of degradation pathways with statistically different abundance in carnivores and herbivores. Pathways in red displayed similar abundance in the metagenomes of carnivores and herbivores.

To further explore possible differential expression of metabolic pathways and GHs showing comparable abundance in metagenomic data collected from herbivores and carnivores, statistical analyses were extended to include all profiled pathways and GHs (Figure 5a). These analyses of transcriptomics data revealed that in comparison to carnivores, herbivores are characterized by increased transcription of genes encoding a range of GH families involved in plant glycan degradation (Figure 5a). Among the latter, members of GH5 encompass cellulases, members of GH97 include α-glucosidases and α-galactosidases, and enzymes belonging to GH130 are known to be involved in the breakdown of β-mannosides such as β-1,4-mannobiose. Furthermore, a range of degradation pathways involved in the metabolism of pectin, including its metabolites 4-deoxy-L-threo-hex-4-enopyranuronate, D-galacturonate and D-fructuronate, as well as the cell wall component L-rhamnose showed higher expression in herbivores (Figure 5a), despite comparable abundance of their corresponding genes in metagenomic data sets of carnivores. In addition, the super-pathway of methanogenesis showed higher expression in herbivores (Figure 5b), possibly reflecting the major metabolic role exerted by methanogens in this class of mammals (Enzmann *et al.*, 2018).

Notably, metatranscriptome data allowed us to confirm functional data obtained from metagenomics approaches and provide insights into the transcriptional profiles of the gut microbial community of herbivores and carnivores in response to the availability of specific dietary components. These findings may support the notion that intestinal microbial populations are able to differentially express genes to maximize food energy/nutrient extraction.

Exploration of functional specialization of the gut microbiome in classes of herbivores

Mammalian fecal samples that had been assessed by shotgun metagenome sequencing were selected to cover the four main classes of herbivores depicted by analysis of 16S rRNA gene microbial profiling data, i.e. polygastric ruminants, polygastric pseudo-ruminants (Tylopoda), heavier monogastric herbivores (>100 Kg of average body weight) and lighter monogastric herbivores (<100 Kg of average body weight). Notably, comparison of the gut microbiome of polygastric ruminants

and pseudo-ruminants revealed very limited differences in terms of encoded pathways and predicted glycobiome (Table S8 and Table S9). In detail, only one metabolic pathway with relative abundance of >0.001 % was found to show increased abundance in ruminants ±50 % compared to pseudo-ruminants (Student's t-test p-value <0.05), i.e. L-glutamate degradation IX (+72.89 %) (Table S8). Moreover, no degradation pathway classes showed statistically significant differential abundance. Notably, these data are consistent with the previously proposed notion that the gut microbiota of these two families of herbivores with a similar multi-chambered digestive system may exert comparable metabolic functions (Dehority, 2002, Al-Masaudi *et al.*, 2019). Indeed, comparison of the number of pathways with a statistically significant different abundance between the two groups of monogastric herbivores and ruminants or pseudo-ruminants revealed similar trends with the only exception of a slight decrease in the number of pathways with statistically significant higher abundance in the pseudo-ruminants than in monogastric herbivores (Table S10). For this reason, ruminants and pseudo-ruminants were considered as a single group for further comparison with heavier monogastric and lighter monogastric herbivores. Metabolic pathway prediction revealed that the total number of pathways with an abundance of >0.001 % and the number of degradative pathways with an abundance of >0.001 % is lower in polygastric animals than in monogastric herbivores.

Furthermore, our collected data revealed that the gut microbiota of ruminants and pseudo-ruminants encodes the highest number of pathways with significant lower abundance in comparison to monogastric herbivores (Figure 6), with a similar trend observed for degradative pathways (Figure 6). A possible explanation for these results is that the higher complexity of the digestive system of polygastric herbivores requires less participation of gut microbiota in the associated catabolic processes than does the situation in monogastric mammals.

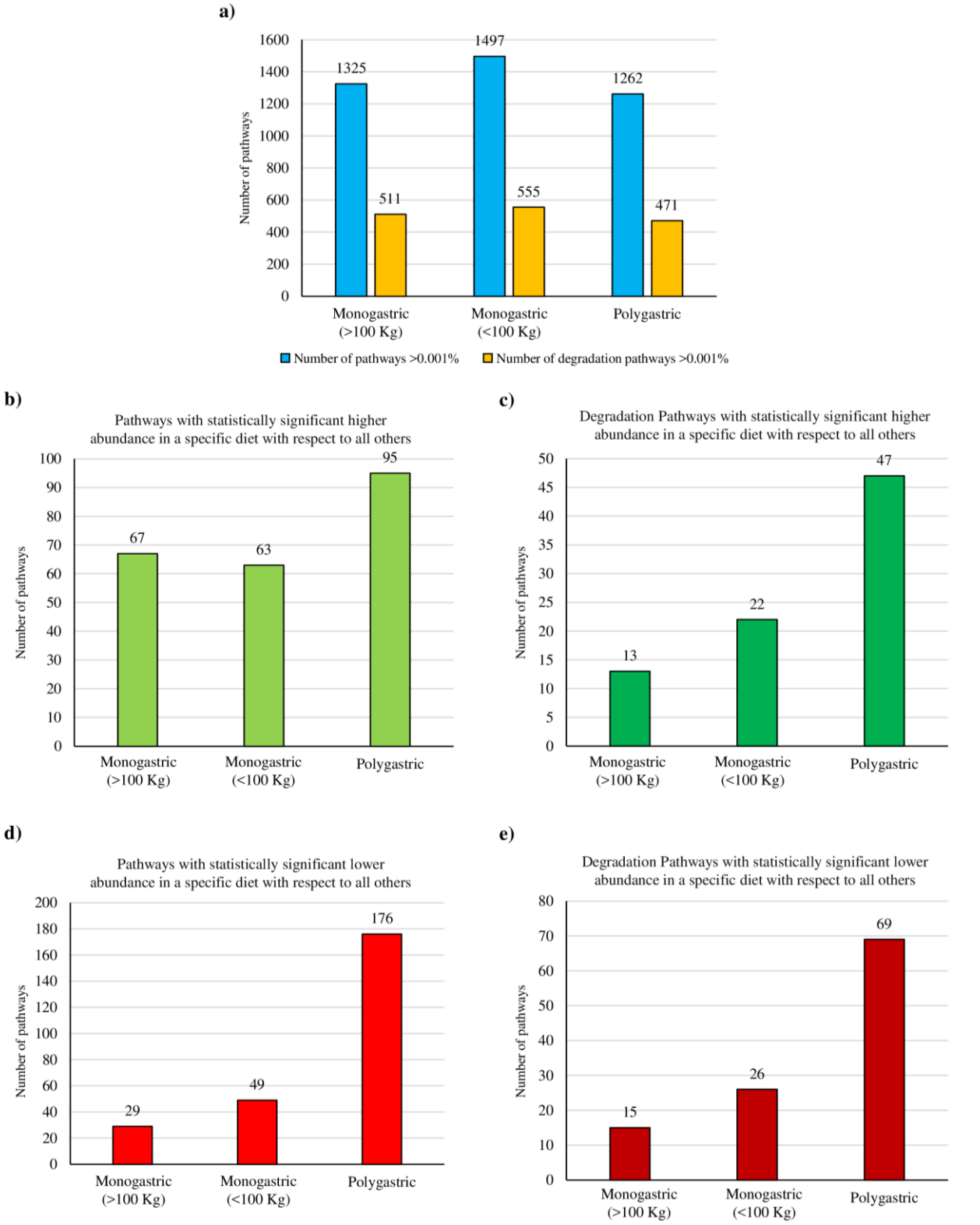


Figure 6. Metabolic pathways prediction in Lighter Monogastric, Heavier Monogastric and Polygastric herbivores. Panel a shows the sum of the number of pathways detected with an abundance of >0.001 %. Panels b and c report the sum of the number of all pathways and degradative pathways with significantly higher abundance in a specific class of herbivores. Panels d and e exhibit the sum of the number of all pathways and degradative pathways with significantly lower abundance in a specific class of herbivores. Statistically significant differences were defined by applying the ANOVA post-hoc Bonferroni statistical analysis.

In contrast, the analysis of shotgun metagenomics data showed that the gut microbiota of lighter monogastric mammals encoded a more extensive repertoire of metabolic pathways (Figure 6). At the same time, as indicated above, 16S rRNA gene-based microbial profiling data revealed that lighter monogastric herbivores possess the lowest gut biodiversity among herbivores (p-value < 0.01)(Figure S1b), probably reflecting the limited colon size responsible for their specialization as cecum fermenters (Dehority, 2002). On the basis of these two observations, it can be assumed that the intestinal bacterial community of lighter monogastric mammals compensates its reduced biodiversity by maximizing its metabolic potential in comparison to heavy herbivores with a more complex digestive system.

In order to further explore peculiar catabolic capabilities of the enrolled classes of herbivores, a detailed evaluation of degradative metabolic pathways enriched in a specific class (analysis of variance [ANOVA] post-hoc Bonferroni P value <0.05 compared to either of the other groups) was performed (Table S10). Notably, the gut microbiota of the heavier monogastric herbivores revealed a specific commitment toward degradation of glycerol and a range of aromatic compounds, including plant metabolites, such as 2, 3-dihydroxybenzoate, or environmental pollutants such as catechol, phenol and toluene (Kahru *et al.*, 2002, Kim *et al.*, 2008, Aghapour *et al.*, 2013) (Table S10). In contrast, the gut microbial population of lighter monogastric herbivores showed a specific abundance of pathways involved in the degradation of plant cell walls, including hemicelluloses and their components, such as glucuronoarabinoxylan and galactans, pectin and rhamnogalacturonan, along with reduction of the inorganic compound sulphate into hydrogen sulphide (Table S10). This observation may suggest that the higher biodiversity of heavier monogastric herbivores (Figure S1) supports specialization of gut commensals toward catabolism of a wider range of secondary plant-related compounds, while the less diverse gut microbial populations of lighter monogastric herbivores (Figure S1) appear more specialized to promote efficient utilization of core plant saccharides. Furthermore, when considering polygastric herbivores, in addition to a higher abundance of pathways for degradation of simple sugars (mono- or di-saccharides) such as D- and L-arabinose, fucose,

maltose, melibiose, trehalose and xylose, this herbivore class showed a higher abundance of a wide range of amino acid degradation pathways (Table S10). Notably, these results suggest that the mammalian gut microbiota plays a significant role in performing specific metabolic tasks dependent not only on host diet but also on the physiology of the corresponding digestive system.

Further exploration of the metabolic potential of herbivores through analysis of their glycobiome revealed that the microbiome of lighter monogastric herbivores encodes the highest number of GH families at a significantly higher abundance (Table 3). Furthermore, five of the six GH families enriched in fecal material of lighter monogastric herbivores are predicted eithr to represent chitinase activity (associated with GH19), which participates in the hydrolysis of (1→4)-β-linkages between N-acetyl-D-glucosamine residues in the chitin-derived chitodextrins (GH25 and GH73), induce breakdown of 1,3-β-glucans (GH81) or encode broad spectrum β-glucosidases and β-mannosidases (GH1). In this context, all of these predicted enzymatic activities may suggest a genetic specialization toward degradation of the main fungal cell wall components (Adams, 2004) (Table 3). Moreover, three of the four GH families enriched in heavier monogastric herbivores are involved in xylan degradation (GH54, GH116 and GH120) (Table 3).Therefore, these data may indicate that the gut microbiota of heavier monogastric herbivores has adapted to compensate for the reduced capability of these animals to metabolize complex plant saccharides in comparison to polygastric ruminants. Furthermore, the abundance of GH family 79, which is enriched in polygastric herbivores (by 803 % and 3981 %) in comparison to lighter and heavier monogastric herbivores, respectively (Table 3), is linked to the degradation of proteoglycans (such as arabinogalactan-linked proteins) (Du *et al.*, 1996, Nothnagel, 1997). Therefore, it seems that the gut microbiota of (pseudo)ruminants is involved in maximizing energy extraction from food through improved breakdown of the extracellular matrix of plants.

Together, these data reveal the relevant role of physiology and anatomy of the mammalian digestive system in order to cooperatively achieve optimal energy extraction from the mammal's particular diet.

Table 3: List of GH families with statistically significant higher or lower abundance based on digestive system's physiology.

GH family	Heavier Monogastric	Lighter Monogastric	Polygastric
GH1	0.0066%	**0.0150%**	0.0075%
GH4	0.0056%	0.0038%	**0.0146%**
GH19	0.0001%	**0.0005%**	0.0001%
GH25	0.0041%	**0.0082%**	0.0048%
GH32	0.0048%	**0.0106%**	0.0051%
GH38	0.0051%	0.0039%	**0.0103%**
GH50	**0.0010%**	0.0004%	0.0004%
GH54	**0.0010%**	0.0000%	0.0001%
GH73	0.0067%	**0.0157%**	0.0061%
GH79	0.0001%	0.0003%	**0.0026%**
GH81	0.0000%	**0.0002%**	0.0001%
GH116	**0.0032%**	0.0007%	0.0008%
GH120	**0.0066%**	**0.0012%**	0.0030%

*percentages in bold indicate Bonferroni post-hoc test p-value <0.05 when compared to other groups.

Chapter 8

General Conclusions

Advances in the characterization of the gut microbiota of dogs and cats

Among the first animals to have been subjected to profound anthropogenic influences, in the course of evolution, dogs and cats have gained a special position in human society. They first became the principal companion animals for humans, while they then were promoted to genuine family members due to their ever closer relationship with their owners. As member of the family, the interest in canine and feline health and well-being has exponentially increased in the last decades with particular attention on the impact that the gut microbiota may have on canine and feline health. In this context, 16S rRNA gene microbial profiling was applied in order to provide an accurate overview of the intestinal microbial community of dogs and cats, allowing to delineate the canine and feline gut microbiota as well as the identification of Gut Community State Types. Both these analyses, coupled with a beta-diversity investigation, revealed differences in the taxonomical composition and biodiversity of the canine and feline gut microbiota, even though these intestinal ecosystems were both dominated by the *Bacteroides*, *Fusobacterium* and *Prevotella* 9 genera, suggesting that these microbial taxa are major players of the intestinal ecosystem of the two abovementioned pets.

Furthermore, bifidobacteria have been described as commensal and abundant microorganisms that inhabit the gastrointestinal tract of animals providing parental care to their offspring, including dogs and cats. Nevertheless, the canine and feline bifidobacterial population has never been fully characterized except through the exclusive use of culture-dependent methods that, as mentioned above, have huge limitation since they rely on growth media and laboratory conditions that are not able to reproduce the complexity of the intestinal environment. Therefore, application of bifidobacterial ITS microbial profiling to canine and feline fecal samples helped to obtain in depth insight into the autochthonous bifidobacterial community of these two companion animals. Specifically, confirming what was observed at the genus level, this approach allowed to reconstruct the bifidobacterial core gut microbiota and to divide fecal samples of dogs and cats into BGSTs

highlighting differences in the bifidobacterial population in term of number of species and taxonomic composition that was further supported by covariance network analyses, thus suggesting an extensive adaptation of the bifidobacterial species to colonize a specific host.

Identification of factors affecting the mammalian gut microbiota composition and functionality

In order to shed light on the factors that are responsible for the modulation of the taxonomical composition and functional activities of the mammalian gut microbiota, a multi-omics approach was applied to a large spectrum of mammalian fecal samples. In detail, considering stool samples from different mammalian species covering the various branches of the tree of life, it has been observed that host physiology and phylogeny, diet as well as anthropogenic influences play crucial roles in driving the mammalian gut microbiome assembly. Specifically, considering the comparison of the 16S rRNA gene microbial profiling between carnivores, herbivores and omnivores, differences in the bacterial biodiversity and the existence of diet-associated genera were demonstrated and the observation that bacterial taxa related to a specific diet co-occur in the mammalian gut ecosystem supports this notion (chapter 7). These findings were further corroborated by the case study involving characterization of the canine gut microbiota. Indeed, comparison of the intestinal microbial community of dogs fed on a BARF diet with that of dogs fed with a commercial food-based diet evidences statistically significant alterations of the relative abundance of diet-associated bacterial genera, such as the saccharolytic *Faecalibacterium* and *Prevotella* which were shown to display higher abundance in the commercial food fed dogs whose diet is enriched in complex carbohydrates (chapter 4). These taxonomic differences also correspond to variations in the genetic repertoire of the intestinal microbial population of mammals following a diverse diet. In this context, shotgun metagenomics revealed a larger variety of genes encoding for enzymes involved in the breakdown of complex plant-derived glycans in herbivores. A similar trend was observed in dogs fed with commercial food when compared to those fed on a BARF diet. The observations may underscore the plasticity of the intestinal microbial community of mammals that seem to have evolved in order to ensure the most efficient energy extraction from diet.

In addition to diet, the host digestive system anatomy was also shown to play a prominent role in modulating both the composition and the metabolic repertoire of the mammalian gut microbiota. Indeed, not only differences were observed in the biodiversity and taxonomical composition when considering the physiology of the mammalian digestive system, but also in the metabolic repertoire since different digestive system physiology corresponds to diverse microbial metabolic capabilities (chapter 7).

Finally, the combination of 16S rRNA gene and bifidobacterial ITS microbial profiling applied to a selection of mammalian species in order to obtain dyads composed by domesticated animals and their feral relatives, highlighted that artificial selection, close contact with humans and the subsequent changes in lifestyle and dietary habits due to domestication have favoured the modulation of the gut microbiota of domesticated mammals. Specifically, the acquisition of microbial taxa linked to a more efficient conversion of feed into body mass and an increase of genes dedicated to the degradation of complex carbohydrates of which animal commercial feeds are enriched suggest an extensive adaptation of the intestinal microbial community of domesticated mammals to their modified lifestyle when compared to their feral counterparts (chapter 6). These observations were further substantiated by the observation that the canine gut microbiota seems to have simultaneously acquired certain bacterial taxa typical of the human intestinal community while they may have lost some bacterial genera characterizing the intestinal ecosystem of their wild relatives, the grey wolves (Chapter 4).

References

Abubucker S, Segata N, Goll J*, et al.* (2012) Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput Biol* **8**: e1002358.

Adams DJ (2004) Fungal cell wall chitinases and glucanases. *Microbiology (Reading)* **150**: 2029-2035.

Agans R, Gordon A, Kramer DL, Perez-Burillo S, Rufian-Henares JA & Paliy O (2018) Dietary Fatty Acids Sustain the Growth of the Human Gut Microbiota. *Appl Environ Microbiol* **84**.

Aghapour AA, Moussavi G & Yaghmaeian K (2013) Biological degradation of catechol in wastewater using the sequencing continuous-inflow reactor (SCR). *J Environ Health Sci Eng* **11**: 3.

Al-Masaudi S, El Kaoutari A, Drula E, Redwan EM, Lombard V & Henrissat B (2019) A metagenomics investigation of carbohydrate-active enzymes along the goat and camel intestinal tract. *Int Microbiol* **22**: 429-435.

Alcon-Giner C, Caim S, Mitra S, Ketskemety J, Wegmann U, Wain J, Belteki G, Clarke P & Hall LJ (2017) Optimisation of 16S rRNA gut microbiota profiling of extremely low birth weight infants. *BMC Genomics* **18**: 841.

Alessandri G, Milani C, Mancabelli L*, et al.* (2019) Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features. *Environ Microbiol* **21**: 1331-1343.

Alvarez-Martin P, Fernandez M, O'Connell-Motherway M, O'Connell KJ, Sauvageot N, Fitzgerald GF, MacSharry J, Zomer A & van Sinderen D (2012) A conserved two-component signal transduction system controls the response to phosphate starvation in Bifidobacterium breve UCC2003. *Appl Environ Microbiol* **78**: 5258-5269.

Arboleya S, Watkins C, Stanton C & Ross RP (2016) Gut Bifidobacteria Populations in Human Health and Aging. *Front Microbiol* **7**: 1204.

Arumugam M, Raes J, Pelletier E*, et al.* (2011) Enterotypes of the human gut microbiome. *Nature* **473**: 174-180.

Aw W & Fukuda S (2015) An Integrated Outlook on the Metagenome and Metabolome of Intestinal Diseases. *Diseases* **3**: 341-359.

Backhed F, Ley RE, Sonnenburg JL, Peterson DA & Gordon JI (2005) Host-bacterial mutualism in the human intestine. *Science* **307**: 1915-1920.

Barker SB & Wolen AR (2008) The benefits of human-companion animal interaction: a review. *J Vet Med Educ* **35**: 487-495.

Barko PC, McMichael MA, Swanson KS & Williams DA (2018) The Gastrointestinal Microbiome: A Review. *J Vet Intern Med* **32**: 9-25.

Barry KA, Wojcicki BJ, Middelbos IS, Vester BM, Swanson KS & Fahey GC, Jr. (2010) Dietary cellulose, fructooligosaccharides, and pectin modify fecal protein catabolites and microbial populations in adult cats. *J Anim Sci* **88**: 2978-2987.

Bartges J, Boynton B, Vogt AH, Krauter E, Lambrecht K, Svec R & Thompson S (2012) AAHA canine life stage guidelines. *J Am Anim Hosp Assoc* **48**: 1-11.

Bashiardes S, Zilberman-Schapira G & Elinav E (2016) Use of Metatranscriptomics in Microbiome Research. *Bioinform Biol Insights* **10**: 19-25.

Bastian M, Heymann, S. and Jacomy, M. (2009) Gephi: an open source software for exploring and manipulating networks. . *International AAAI Conference on Weblogs and Social Media*.

Behjati S & Tarpey PS (2013) What is next generation sequencing? *Arch Dis Child Educ Pract Ed* **98**: 236-238.

Belzer C & de Vos WM (2012) Microbes inside--from diversity to function: the case of Akkermansia. *ISME J* **6**: 1449-1458.

Benson AK (2015) Host genetic architecture and the landscape of microbiome composition: humans weigh in. *Genome Biol* **16**: 203.
Bermingham EN, Maclean P, Thomas DG, Cave NJ & Young W (2017) Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs. *PeerJ* **5**: e3019.
Biavati B, Scardovi V & C. MwE (1982) Electrophoretic Patterns of Proteins in the Genus Bifidobacterium and Proposal of Four New Species.
Biteen JS, Blainey PC, Cardon ZG*, et al.* (2016) Tools for the Microbiome: Nano and Beyond. *ACS Nano* **10**: 6-37.
Bittar F, Keita MB, Lagier JC, Peeters M, Delaporte E & Raoult D (2014) Gorilla gorilla gorilla gut: a potential reservoir of pathogenic bacteria as revealed using culturomics and molecular tools. *Sci Rep* **4**: 7174.
Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA & Gregory Caporaso J (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**: 90.
Bottacini F, van Sinderen D & Ventura M (2017) Omics of bifidobacteria: research and insights into their health-promoting activities. *Biochem J* **474**: 4137-4152.
Bottacini F, Ventura M, van Sinderen D & O'Connell Motherway M (2014) Diversity, ecology and intestinal function of bifidobacteria. *Microb Cell Fact* **13 Suppl 1**: S4.
Bottacini F, Milani C, Turroni F*, et al.* (2012) Bifidobacterium asteroides PRL2011 genome analysis reveals clues for colonization of the insect gut. *PLoS One* **7**: e44229.
Bottari B, Ercolini D, Gatti M & Neviani E (2006) Application of FISH technology for microbiological analysis: current state and prospects. *Appl Microbiol Biotechnol* **73**: 485-494.
Brooks AW, Kohl KD, Brucker RM, van Opstal EJ & Bordenstein SR (2016) Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History. *PLoS Biol* **14**: e2000225.
Bull JW & Maron M (2016) How humans drive speciation as well as extinction. *Proc Biol Sci* **283**.
Bunesova V, Vlkova E, Rada V, Killer J & Musilova S (2014) Bifidobacteria from the gastrointestinal tract of animals: differences and similarities. *Benef Microbes* **5**: 377-388.
Butowski CF, Thomas DG, Young W, Cave NJ, McKenzie CM, Rosendale DI & Bermingham EN (2019) Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal bacteriome and organic acid profiles of the domestic cat (Felis catus). *PLoS One* **14**: e0216072.
Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ & Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**: 581-583.
Caporaso JG, Kuczynski J, Stombaugh J*, et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.
Carmona M, Zamarro MT, Blazquez B, Durante-Rodriguez G, Juarez JF, Valderrama JA, Barragan MJ, Garcia JL & Diaz E (2009) Anaerobic catabolism of aromatic compounds: a genetic and genomic view. *Microbiol Mol Biol Rev* **73**: 71-133.
Carrillo-Araujo M, Tas N, Alcantara-Hernandez RJ, Gaona O, Schondube JE, Medellin RA, Jansson JK & Falcon LI (2015) Phyllostomid bat microbiome composition is associated to host phylogeny and feeding strategies. *Front Microbiol* **6**: 447.
Caspi R, Foerster H, Fulcher CA*, et al.* (2008) The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic Acids Res* **36**: D623-631.
Caspi R, Billington R, Ferrer L*, et al.* (2016) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **44**: D471-480.
Castellarin M, Warren RL, Freeman JD*, et al.* (2012) Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* **22**: 299-306.
Cohen SP (2002) Can pets function as family members? *West J Nurs Res* **24**: 621-638.
Coyte KZ & Rakoff-Nahoum S (2019) Understanding Competition and Cooperation within the Mammalian Gut Microbiome. *Curr Biol* **29**: R538-R544.

Crost EH, Le Gall G, Laverde-Gomez JA, Mukhopadhya I, Flint HJ & Juge N (2018) Mechanistic Insights Into the Cross-Feeding of Ruminococcus gnavus and Ruminococcus bromii on Host and Dietary Carbohydrates. *Front Microbiol* **9**: 2558.
Davenport ER, Sanders JG, Song SJ, Amato KR, Clark AG & Knight R (2017) The human microbiome in evolution. *BMC Biol* **15**: 127.
David LA, Maurice CF, Carmody RN*, et al.* (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**: 559-563.
De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G & Lionetti P (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A* **107**: 14691-14696.
de Godoy MR, Kerr KR & Fahey GC, Jr. (2013) Alternative dietary fiber sources in companion animal nutrition. *Nutrients* **5**: 3099-3117.
De Vuyst L, Moens F, Selak M, Riviere A & Leroy F (2014) Summer Meeting 2013: growth and physiology of bifidobacteria. *J Appl Microbiol* **116**: 477-491.
Dearing MD & Kohl KD (2017) Beyond Fermentation: Other Important Services Provided to Endothermic Herbivores by their Gut Microbiota. *Integr Comp Biol* **57**: 723-731.
Dehority BA (2002) Gastrointestinal tracts of herbivores, particularly the ruminant: Anatomy, physiology and microbial digestion of plants. *J Appl Anim Res* **21**: 145-160.
Delcenserie V, Gavini F, Beerens H, Tresse O, Franssen C & Daube G (2007) Description of a new species, Bifidobacterium crudilactis sp. nov., isolated from raw milk and raw milk cheeses. *Syst Appl Microbiol* **30**: 381-389.
Delsuc F, Metcalf JL, Wegener Parfrey L, Song SJ, Gonzalez A & Knight R (2014) Convergence of gut microbiomes in myrmecophagous mammals. *Mol Ecol* **23**: 1301-1317.
den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ & Bakker BM (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* **54**: 2325-2340.
Deng P & Swanson KS (2015) Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *Br J Nutr* **113 Suppl**: S6-17.
Diaz Heijtz R, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, Hibberd ML, Forssberg H & Pettersson S (2011) Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* **108**: 3047-3052.
Doron L, Coppenhagen-Glazer S, Ibrahim Y, Eini A, Naor R, Rosen G & Bachrach G (2014) Identification and characterization of fusolisin, the Fusobacterium nucleatum autotransporter serine protease. *PLoS One* **9**: e111329.
Dotson MJ & Hyatt EM (2008) Understanding dog-human companionship. *Journal of Business Research* **61**: 457-466.
Driscoll CA, Macdonald DW & O'Brien SJ (2009) From wild animals to domestic pets, an evolutionary view of domestication. *Proc Natl Acad Sci U S A* **106 Suppl 1**: 9971-9978.
Du H, Clarke AE & Bacic A (1996) Arabinogalactan-proteins: a class of extracellular matrix proteoglycans involved in plant growth and development. *Trends Cell Biol* **6**: 411-414.
Duranti S, Mangifesta M, Lugli GA, Turroni F, Anzalone R, Milani C, Mancabelli L, Ossiprandi MC & Ventura M (2017) Bifidobacterium vansinderenii sp. nov., isolated from faeces of emperor tamarin (Saguinus imperator). *Int J Syst Evol Microbiol* **67**: 3987-3995.
Duranti S, Turroni F, Lugli GA, Milani C, Viappiani A, Mangifesta M, Gioiosa L, Palanza P, van Sinderen D & Ventura M (2014) Genomic characterization and transcriptional studies of the starch-utilizing strain Bifidobacterium adolescentis 22L. *Appl Environ Microbiol* **80**: 6080-6090.
Duranti S, Lugli GA, Milani C*, et al.* (2019) Bifidobacterium bifidum and the infant gut microbiota: an intriguing case of microbe-host co-evolution. *Environ Microbiol*.
Duranti S, Lugli GA, Mancabelli L*, et al.* (2017) Maternal inheritance of bifidobacterial communities and bifidophages in infants through vertical transmission. *Microbiome* **5**: 66.

Egan M, O'Connell Motherway M, Ventura M & van Sinderen D (2014) Metabolism of sialic acid by Bifidobacterium breve UCC2003. *Appl Environ Microbiol* **80**: 4414-4426.
Enzmann F, Mayer F, Rother M & Holtmann D (2018) Methanogens: biochemical background and biotechnological applications. *AMB Express* **8**: 1.
Fanning S, Hall LJ, Cronin M*, et al.* (2012) Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proc Natl Acad Sci U S A* **109**: 2108-2113.
Ferrario C, Alessandri G, Mancabelli L*, et al.* (2017) Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses. *Environ Microbiol* **19**: 4771-4783.
Finlayson-Trick ECL, Getz LJ, Slaine PD*, et al.* (2017) Taxonomic differences of gut microbiomes drive cellulolytic enzymatic potential within hind-gut fermenting mammals. *PLoS One* **12**: e0189404.
Fleming JM, Creevy KE & Promislow DE (2011) Mortality in north american dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *J Vet Intern Med* **25**: 187-198.
Flint HJ, Bayer EA, Rincon MT, Lamed R & White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol* **6**: 121-131.
Flint HJ, Scott KP, Duncan SH, Louis P & Forano E (2012) Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**: 289-306.
Foroni E, Serafini F, Amidani D*, et al.* (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium. *Microb Cell Fact* **10 Suppl 1**: S16.
Fredriksson-Ahomaa M, Heikkila T, Pernu N, Kovanen S, Hielm-Bjorkman A & Kivisto R (2017) Raw Meat-Based Diets in Dogs and Cats. *Vet Sci* **4**.
Freeman LM, Chandler ML, Hamper BA & Weeth LP (2013) Current knowledge about the risks and benefits of raw meat-based diets for dogs and cats. *J Am Vet Med Assoc* **243**: 1549-1558.
Furrie E (2006) A molecular revolution in the study of intestinal microflora. *Gut* **55**: 141-143.
Gagniere J, Raisch J, Veziant J, Barnich N, Bonnet R, Buc E, Bringer MA, Pezet D & Bonnet M (2016) Gut microbiota imbalance and colorectal cancer. *World J Gastroenterol* **22**: 501-518.
Garcia-Bayona L & Comstock LE (2018) Bacterial antagonism in host-associated microbial communities. *Science* **361**.
Glickman ME, Rao SR & Schultz MR (2014) False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies. *J Clin Epidemiol* **67**: 850-857.
Gloor GB, Macklaim JM, Pawlowsky-Glahn V & Egozcue JJ (2017) Microbiome Datasets Are Compositional: And This Is Not Optional. *Front Microbiol* **8**: 2224.
Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, Dantas G & Gordon JI (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proc Natl Acad Sci U S A* **108**: 6252-6257.
Greer KA, Canterberry SC & Murphy KE (2007) Statistical analysis regarding the effects of height and weight on life span of the domestic dog. *Res Vet Sci* **82**: 208-214.
Greub G (2012) Culturomics: a new approach to study the human microbiome. *Clin Microbiol Infect* **18**: 1157-1159.
Group NHW, Peterson J, Garges S*, et al.* (2009) The NIH Human Microbiome Project. *Genome Res* **19**: 2317-2323.
Groussin M, Mazel F, Sanders JG, Smillie CS, Lavergne S, Thuiller W & Alm EJ (2017) Unraveling the processes shaping mammalian gut microbiomes over evolutionary time. *Nat Commun* **8**: 14319.
Grzeskowiak L, Endo A, Beasley S & Salminen S (2015) Microbiota and probiotics in canine and feline welfare. *Anaerobe* **34**: 14-23.
Gudeta DD, Bortolaia V, Amos G, Wellington EM, Brandt KK, Poirel L, Nielsen JB, Westh H & Guardabassi L (2016) The Soil Microbiota Harbors a Diversity of Carbapenem-Hydrolyzing beta-Lactamases of Potential Clinical Relevance. *Antimicrob Agents Chemother* **60**: 151-160.

Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilan CG & Margolles A (2009) Bile-inducible efflux transporter from Bifidobacterium longum NCC2705, conferring bile resistance. *Appl Environ Microbiol* **75**: 3153-3160.
Hamady M & Knight R (2009) Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Res* **19**: 1141-1152.
Hand D, Wallis C, Colyer A & Penn CW (2013) Pyrosequencing the canine faecal microbiota: breadth and depth of biodiversity. *PLoS One* **8**: e53115.
Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM & Suchodolski JS (2011) Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol Ecol* **76**: 301-310.
Herstad KMV, Gajardo K, Bakke AM, Moe L, Ludvigsen J, Rudi K, Rud I, Sekelja M & Skancke E (2017) A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. *BMC Vet Res* **13**: 147.
Hidalgo-Cantabrana C, Delgado S, Ruiz L, Ruas-Madiedo P, Sanchez B & Margolles A (2017) Bifidobacteria and Their Health-Promoting Effects. *Microbiol Spectr* **5**.
Hillman ET, Lu H, Yao T & Nakatsu CH (2017) Microbial Ecology along the Gastrointestinal Tract. *Microbes Environ* **32**: 300-313.
Honneffer JB, Minamoto Y & Suchodolski JS (2014) Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. *World J Gastroenterol* **20**: 16489-16497.
Hooda S, Vester Boler BM, Kerr KR, Dowd SE & Swanson KS (2013) The gut microbiome of kittens is affected by dietary protein:carbohydrate ratio and associated with blood metabolite and hormone concentrations. *Br J Nutr* **109**: 1637-1646.
Hoyles L, Inganas E, Falsen E, Drancourt M, Weiss N, McCartney AL & Collins MD (2002) Bifidobacterium scardovii sp. nov., from human sources. *Int J Syst Evol Microbiol* **52**: 995-999.
Hugon P, Dufour JC, Colson P, Fournier PE, Sallah K & Raoult D (2015) A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect Dis* **15**: 1211-1219.
Human Microbiome Project C (2012) Structure, function and diversity of the healthy human microbiome. *Nature* **486**: 207-214.
Integrative HMPRNC (2014) The Integrative Human Microbiome Project: dynamic analysis of microbiome-host omics profiles during periods of human health and disease. *Cell Host Microbe* **16**: 276-289.
Janda JM & Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* **45**: 2761-2764.
Jimenez DJ, de Lima Brossi MJ, Schuckel J, Kracun SK, Willats WG & van Elsas JD (2016) Characterization of three plant biomass-degrading microbial consortia by metagenomics- and metasecretomics-based approaches. *Appl Microbiol Biotechnol* **100**: 10463-10477.
Jones SA, Jorgensen M, Chowdhury FZ, Rodgers R, Hartline J, Leatham MP, Struve C, Krogfelt KA, Cohen PS & Conway T (2008) Glycogen and maltose utilization by Escherichia coli O157:H7 in the mouse intestine. *Infect Immun* **76**: 2531-2540.
Kahru A, Maloverjan A, Sillak H & Pollumaa L (2002) The toxicity and fate of phenolic pollutants in the contaminated soils associated with the oil-shale industry. *Environ Sci Pollut Res Int* **Spec No 1**: 27-33.
Kennedy K, Hall MW, Lynch MD, Moreno-Hagelsieb G & Neufeld JD (2014) Evaluating bias of illumina-based bacterial 16S rRNA gene profiles. *Appl Environ Microbiol* **80**: 5717-5722.
Kil DY & Swanson KS (2011) Companion animals symposium: role of microbes in canine and feline health. *J Anim Sci* **89**: 1498-1505.
Killer J, Kopecny J, Mrazek J, Koppova I, Havlik J, Benada O & Kott T (2011) Bifidobacterium actinocoloniiforme sp. nov. and Bifidobacterium bohemicum sp. nov., from the bumblebee digestive tract. *Int J Syst Evol Microbiol* **61**: 1315-1321.
Kim HJ, Choi SW & Inyang HI (2008) Catalytic oxidation of toluene in contaminant emission control systems using Mn-Ce/gamma-Al2O3. *Environ Technol* **29**: 559-569.

Kim J, An JU, Kim W, Lee S & Cho S (2017) Differences in the gut microbiota of dogs (Canis lupus familiaris) fed a natural diet or a commercial feed revealed by the Illumina MiSeq platform. *Gut Pathog* **9**: 68.
Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M & Glockner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41**: e1.
Koboldt DC, Steinberg KM, Larson DE, Wilson RK & Mardis ER (2013) The next-generation sequencing revolution and its impact on genomics. *Cell* **155**: 27-38.
Kotake T, Yamanashi Y, Imaizumi C & Tsumuraya Y (2016) Metabolism of L-arabinose in plants. *J Plant Res* **129**: 781-792.
La Reau AJ & Suen G (2018) The Ruminococci: key symbionts of the gut ecosystem. *J Microbiol* **56**: 199-208.
Lagier JC, Million M, Hugon P, Armougom F & Raoult D (2012) Human gut microbiota: repertoire and variations. *Front Cell Infect Microbiol* **2**: 136.
Lagier JC, Hugon P, Khelaifia S, Fournier PE, La Scola B & Raoult D (2015) The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin Microbiol Rev* **28**: 237-264.
Leuenberger C, Czuczwa J, Tremp J & Giger W (1988) Nitrated phenols in rain: Atmospheric occurrence of phytotoxic pollutants. *Chemosphere* **17**: 511-515.
Ley RE, Lozupone CA, Hamady M, Knight R & Gordon JI (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* **6**: 776-788.
Ley RE, Hamady M, Lozupone C*, et al.* (2008) Evolution of mammals and their gut microbes. *Science* **320**: 1647-1651.
Li H & Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754-1760.
Lin L & Zhang J (2017) Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol* **18**: 2.
Liu JP, Zou WL, Chen SJ, Wei HY, Yin YN, Zou YY & Lu FG (2016) Effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease development. *World J Gastroenterol* **22**: 7353-7364.
Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM & Henrissat B (2014) The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res* **42**: D490-495.
Lopez-Siles M, Khan TM, Duncan SH, Harmsen HJ, Garcia-Gil LJ & Flint HJ (2012) Cultured representatives of two major phylogroups of human colonic Faecalibacterium prausnitzii can utilize pectin, uronic acids, and host-derived substrates for growth. *Appl Environ Microbiol* **78**: 420-428.
Lopez-Siles M, Enrich-Capo N, Aldeguer X, Sabat-Mir M, Duncan SH, Garcia-Gil LJ & Martinez-Medina M (2018) Alterations in the Abundance and Co-occurrence of Akkermansia muciniphila and Faecalibacterium prausnitzii in the Colonic Mucosa of Inflammatory Bowel Disease Subjects. *Front Cell Infect Microbiol* **8**: 281.
Lugli GA, Milani C, Turroni F*, et al.* (2017) Comparative genomic and phylogenomic analyses of the Bifidobacteriaceae family. *BMC Genomics* **18**: 568.
Lugli GA, Mancino W, Milani C*, et al.* (2019) Dissecting the Evolutionary Development of the Species Bifidobacterium animalis through Comparative Genomics Analyses. *Appl Environ Microbiol* **85**.
Lyu T, Liu G, Zhang H, Wang L, Zhou S, Dou H, Pang B, Sha W & Zhang H (2018) Changes in feeding habits promoted the differentiation of the composition and function of gut microbiotas between domestic dogs (Canis lupus familiaris) and gray wolves (Canis lupus). *AMB Express* **8**: 123.
Mancabelli L, Milani C, Lugli GA*, et al.* (2017) Unveiling the gut microbiota composition and functionality associated with constipation through metagenomic analyses. *Sci Rep* **7**: 9879.

Marmol I, Sanchez-de-Diego C, Pradilla Dieste A, Cerrada E & Rodriguez Yoldi MJ (2017) Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. *Int J Mol Sci* **18**.
Masuoka H, Shimada K, Kiyosue-Yasuda T*, et al.* (2017) Transition of the intestinal microbiota of cats with age. *PLoS One* **12**: e0181739.
Matsuzawa T, Kaneko S & Yaoi K (2015) Screening, identification, and characterization of a GH43 family beta-xylosidase/alpha-arabinofuranosidase from a compost microbial metagenome. *Appl Microbiol Biotechnol* **99**: 8943-8954.
McFall-Ngai M, Hadfield MG, Bosch TC*, et al.* (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci U S A* **110**: 3229-3236.
McKenzie VJ, Song SJ, Delsuc F*, et al.* (2017) The Effects of Captivity on the Mammalian Gut Microbiome. *Integr Comp Biol* **57**: 690-704.
Menni C, Jackson MA, Pallister T, Steves CJ, Spector TD & Valdes AM (2017) Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. *Int J Obes (Lond)* **41**: 1099-1105.
Mentula S, Harmoinen J, Heikkila M, Westermarck E, Rautio M, Huovinen P & Kononen E (2005) Comparison between cultured small-intestinal and fecal microbiotas in beagle dogs. *Appl Environ Microbiol* **71**: 4169-4175.
Metcalf JL, Song SJ, Morton JT*, et al.* (2017) Evaluating the impact of domestication and captivity on the horse gut microbiome. *Sci Rep* **7**: 15497.
Michelini S, Modesto M, Filippini G, Spiezio C, Sandri C, Biavati B, Pisi A & Mattarelli P (2016) Bifidobacterium aerophilum sp. nov., Bifidobacterium avesanii sp. nov. and Bifidobacterium ramosum sp. nov.: Three novel taxa from the faeces of cotton-top tamarin (Saguinus oedipus L.). *Syst Appl Microbiol* **39**: 229-236.
Michelini S, Oki K, Yanokura E, Shimakawa Y, Modesto M, Mattarelli P, Biavati B & Watanabe K (2016) Bifidobacterium myosotis sp. nov., Bifidobacterium tissieri sp. nov. and Bifidobacterium hapali sp. nov., isolated from faeces of baby common marmosets (Callithrix jacchus L.). *Int J Syst Evol Microbiol* **66**: 255-265.
Middelbos IS, Vester Boler BM, Qu A, White BA, Swanson KS & Fahey GC, Jr. (2010) Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. *PLoS One* **5**: e9768.
Milani C, Turroni F, Duranti S, Lugli GA, Mancabelli L, Ferrario C, van Sinderen D & Ventura M (2016) Genomics of the Genus Bifidobacterium Reveals Species-Specific Adaptation to the Glycan-Rich Gut Environment. *Appl Environ Microbiol* **82**: 980-991.
Milani C, Lugli GA, Turroni F, Mancabelli L, Duranti S, Viappiani A, Mangifesta M, Segata N, van Sinderen D & Ventura M (2014) Evaluation of bifidobacterial community composition in the human gut by means of a targeted amplicon sequencing (ITS) protocol. *FEMS Microbiol Ecol* **90**: 493-503.
Milani C, Mangifesta M, Mancabelli L, Lugli GA, Mancino W, Viappiani A, Faccini A, van Sinderen D, Ventura M & Turroni F (2017) The Sortase-Dependent Fimbriome of the Genus Bifidobacterium: Extracellular Structures with Potential To Modulate Microbe-Host Dialogue. *Appl Environ Microbiol* **83**.
Milani C, Mangifesta M, Mancabelli L*, et al.* (2017) Unveiling bifidobacterial biogeography across the mammalian branch of the tree of life. *ISME J* **11**: 2834-2847.
Milani C, Hevia A, Foroni E*, et al.* (2013) Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PLoS One* **8**: e68739.
Milani C, Lugli GA, Duranti S*, et al.* (2014) Genomic encyclopedia of type strains of the genus Bifidobacterium. *Appl Environ Microbiol* **80**: 6290-6302.
Milani C, Casey E, Lugli GA*, et al.* (2018) Tracing mother-infant transmission of bacteriophages by means of a novel analytical tool for shotgun metagenomic datasets: METAnnotatorX. *Microbiome* **6**: 145.

Milani C, Duranti S, Bottacini F*, et al.* (2017) The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev* **81**.
Milani C, Lugli GA, Duranti S*, et al.* (2015) Bifidobacteria exhibit social behavior through carbohydrate resource sharing in the gut. *Sci Rep* **5**: 15782.
Minamoto Y, Hooda S, Swanson KS & Suchodolski JS (2012) Feline gastrointestinal microbiota. *Anim Health Res Rev* **13**: 64-77.
Miyazaki K, Hirase T, Kojima Y & Flint HJ (2005) Medium- to large-sized xylo-oligosaccharides are responsible for xylanase induction in Prevotella bryantii B14. *Microbiology* **151**: 4121-4125.
Modesto M, Michelini S, Stefanini I, Sandri C, Spiezio C, Pisi A, Filippini G, Biavati B & Mattarelli P (2015) Bifidobacterium lemurum sp. nov., from faeces of the ring-tailed lemur (Lemur catta). *Int J Syst Evol Microbiol* **65**: 1726-1734.
Moens E & Veldhoen M (2012) Epithelial barrier biology: good fences make good neighbours. *Immunology* **135**: 1-8.
Moon CD, Young W, Maclean PH, Cookson AL & Bermingham EN (2018) Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiologyopen* **7**: e00677.
Moran NA (2006) Symbiosis. *Curr Biol* **16**: R866-871.
Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L, Henrissat B, Knight R & Gordon JI (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* **332**: 970-974.
Nelson TM, Rogers TL, Carlini AR & Brown MV (2013) Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ Microbiol* **15**: 1132-1145.
Nicholson JK, Holmes E & Wilson ID (2005) Gut microorganisms, mammalian metabolism and personalized health care. *Nat Rev Microbiol* **3**: 431-438.
Nothnagel EA (1997) Proteoglycans and related components in plant cells. *Int Rev Cytol* **174**: 195-291.
O'Callaghan A & van Sinderen D (2016) Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front Microbiol* **7**: 925.
O'Connell Motherway M, Zomer A, Leahy SC*, et al.* (2011) Functional genome analysis of Bifidobacterium breve UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc Natl Acad Sci U S A* **108**: 11217-11222.
Okamoto M, Benno Y, Leung KP & Maeda N (2008) Bifidobacterium tsurumiense sp. nov., from hamster dental plaque. *Int J Syst Evol Microbiol* **58**: 144-148.
Parker HG, Kim LV, Sutter NB, Carlson S, Lorentzen TD, Malek TB, Johnson GS, DeFrance HB, Ostrander EA & Kruglyak L (2004) Genetic structure of the purebred domestic dog. *Science* **304**: 1160-1164.
Perea K, Perz K, Olivo SK, Williams A, Lachman M, Ishaq SL, Thomson J & Yeoman CJ (2017) Feed efficiency phenotypes in lambs involve changes in ruminal, colonic, and small-intestine-located microbiota. *J Anim Sci* **95**: 2585-2592.
Perez PF, Minnaard Y, Disalvo EA & De Antoni GL (1998) Surface properties of bifidobacterial strains of human origin. *Appl Environ Microbiol* **64**: 21-26.
Phillips CD, Phelan G, Dowd SE, McDonough MM, Ferguson AW, Delton Hanson J, Siles L, Ordonez-Garza N, San Francisco M & Baker RJ (2012) Microbiome analysis among bats describes influences of host phylogeny, life history, physiology and geography. *Mol Ecol* **21**: 2617-2627.
Pickard JM, Zeng MY, Caruso R & Nunez G (2017) Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev* **279**: 70-89.
Pinna C & Biagi G (2016) The Utilisation of Prebiotics and Synbiotics in Dogs. *Italian Journal of Animal Science* **13**.

Pokusaeva K, Fitzgerald GF & van Sinderen D (2011) Carbohydrate metabolism in Bifidobacteria. *Genes Nutr* **6**: 285-306.
Poretsky RS, Hewson I, Sun S, Allen AE, Zehr JP & Moran MA (2009) Comparative day/night metatranscriptomic analysis of microbial communities in the North Pacific subtropical gyre. *Environ Microbiol* **11**: 1358-1375.
Potempa J, Korzus E & Travis J (1994) The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J Biol Chem* **269**: 15957-15960.
Potrykus J, White RL & Bearne SL (2008) Proteomic investigation of amino acid catabolism in the indigenous gut anaerobe Fusobacterium varium. *Proteomics* **8**: 2691-2703.
Praet J, Meeus I, Cnockaert M, Aerts M, Smagghe G & Vandamme P (2015) Bifidobacterium commune sp. nov. isolated from the bumble bee gut. *Antonie Van Leeuwenhoek* **107**: 1307-1313.
Price CE, Reid SJ, Driessen AJ & Abratt VR (2006) The Bifidobacterium longum NCIMB 702259T ctr gene codes for a novel cholate transporter. *Appl Environ Microbiol* **72**: 923-926.
Qin J, Li R, Raes J*, et al.* (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**: 59-65.
Quan J, Cai G, Ye J*, et al.* (2018) A global comparison of the microbiome compositions of three gut locations in commercial pigs with extreme feed conversion ratios. *Sci Rep* **8**: 4536.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J & Glockner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**: D590-596.
Quince C, Walker AW, Simpson JT, Loman NJ & Segata N (2017) Shotgun metagenomics, from sampling to analysis. *Nat Biotechnol* **35**: 833-844.
Rajilic-Stojanovic M & de Vos WM (2014) The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiol Rev* **38**: 996-1047.
Rath S, Heidrich B, Pieper DH & Vital M (2017) Uncovering the trimethylamine-producing bacteria of the human gut microbiota. *Microbiome* **5**: 54.
Rinke C, Schwientek P, Sczyrba A*, et al.* (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**: 431-437.
Ritchie LE, Steiner JM & Suchodolski JS (2008) Assessment of microbial diversity along the feline intestinal tract using 16S rRNA gene analysis. *FEMS Microbiol Ecol* **66**: 590-598.
Ritchie LE, Burke KF, Garcia-Mazcorro JF, Steiner JM & Suchodolski JS (2010) Characterization of fecal microbiota in cats using universal 16S rRNA gene and group-specific primers for Lactobacillus and Bifidobacterium spp. *Vet Microbiol* **144**: 140-146.
Rochus K, Janssens GP & Hesta M (2014) Dietary fibre and the importance of the gut microbiota in feline nutrition: a review. *Nutr Res Rev* **27**: 295-307.
Ruas-Madiedo P, Gueimonde M, Arigoni F, de los Reyes-Gavilan CG & Margolles A (2009) Bile affects the synthesis of exopolysaccharides by Bifidobacterium animalis. *Appl Environ Microbiol* **75**: 1204-1207.
Ruas M, Salazar N & De Los Reyes-Gavilan CG (2009) Biosynthesis and Chemical Composition of Exopolysaccharides Produced by Lactic Acid Bacteria. *Bacterial Polysaccharides: Current Innovations and Future Trends*.
Ruiz L, Zomer A, O'Connell-Motherway M, van Sinderen D & Margolles A (2012) Discovering novel bile protection systems in Bifidobacterium breve UCC2003 through functional genomics. *Appl Environ Microbiol* **78**: 1123-1131.
Ruiz L, Delgado S, Ruas-Madiedo P, Sanchez B & Margolles A (2017) Bifidobacteria and Their Molecular Communication with the Immune System. *Front Microbiol* **8**: 2345.
Ruiz L, O'Connell-Motherway M, Zomer A, de los Reyes-Gavilan CG, Margolles A & van Sinderen D (2012) A bile-inducible membrane protein mediates bifidobacterial bile resistance. *Microb Biotechnol* **5**: 523-535.
Russell JB & Rychlik JL (2001) Factors that alter rumen microbial ecology. *Science* **292**: 1119-1122.

Sabbioni A, Ferrario C, Milani C, Mancabelli L, Riccardi E, Di Ianni F, Beretti V, Superchi P & Ossiprandi MC (2016) Modulation of the Bifidobacterial Communities of the Dog Microbiota by Zeolite. *Front Microbiol* **7**: 1491.
Salonen A, Salojarvi J, Lahti L & de Vos WM (2012) The adult intestinal core microbiota is determined by analysis depth and health status. *Clin Microbiol Infect* **18 Suppl 4**: 16-20.
Sanders JG, Powell S, Kronauer DJ, Vasconcelos HL, Frederickson ME & Pierce NE (2014) Stability and phylogenetic correlation in gut microbiota: lessons from ants and apes. *Mol Ecol* **23**: 1268-1283.
Sanders JG, Beichman AC, Roman J, Scott JJ, Emerson D, McCarthy JJ & Girguis PR (2015) Baleen whales host a unique gut microbiome with similarities to both carnivores and herbivores. *Nat Commun* **6**: 8285.
Savolainen P, Zhang YP, Luo J, Lundeberg J & Leitner T (2002) Genetic evidence for an East Asian origin of domestic dogs. *Science* **298**: 1610-1613.
Schell MA, Karmirantzou M, Snel B*, et al.* (2002) The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci U S A* **99**: 14422-14427.
Schlesinger DP & Joffe DJ (2011) Raw food diets in companion animals: a critical review. *Can Vet J* **52**: 50-54.
Schmidt M, Unterer S, Suchodolski JS, Honneffer JB, Guard BC, Lidbury JA, Steiner JM, Fritz J & Kolle P (2018) The fecal microbiome and metabolome differs between dogs fed Bones and Raw Food (BARF) diets and dogs fed commercial diets. *PLoS One* **13**: e0201279.
Schnorr SL, Candela M, Rampelli S*, et al.* (2014) Gut microbiome of the Hadza hunter-gatherers. *Nat Commun* **5**: 3654.
Scupham AJ, Patton TG, Bent E & Bayles DO (2008) Comparison of the cecal microbiota of domestic and wild turkeys. *Microb Ecol* **56**: 322-331.
Segarra S, Martinez-Subiela S, Cerda-Cuellar M, Martinez-Puig D, Munoz-Prieto A, Rodriguez-Franco F, Rodriguez-Bertos A, Allenspach K, Velasco A & Ceron J (2016) Oral chondroitin sulfate and prebiotics for the treatment of canine Inflammatory Bowel Disease: a randomized, controlled clinical trial. *BMC Vet Res* **12**: 49.
Segata N, Boernigen D, Tickle TL, Morgan XC, Garrett WS & Huttenhower C (2013) Computational meta'omics for microbial community studies. *Mol Syst Biol* **9**: 666.
Sender R, Fuchs S & Milo R (2016) Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* **14**: e1002533.
Sender R, Fuchs S & Milo R (2016) Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell* **164**: 337-340.
Slavin J (2013) Fiber and prebiotics: mechanisms and health benefits. *Nutrients* **5**: 1417-1435.
Sokol H, Pigneur B, Watterlot L*, et al.* (2008) Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* **105**: 16731-16736.
Stevens C & Hume I (2004) Comparative physiology of the vertebrate digestive system
Suchodolski JS (2016) Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. *Vet J* **215**: 30-37.
Suchodolski JS, Camacho J & Steiner JM (2008) Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. *FEMS Microbiol Ecol* **66**: 567-578.
Sun Y, Zhao Q, Zhi D*, et al.* (2017) Sporosarcina terrae sp. nov., isolated from orchard soil. *Int J Syst Evol Microbiol* **67**: 2104-2108.
Suzuki MT & Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62**: 625-630.
Suzuki TA (2017) Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. *Integr Comp Biol* **57**: 756-769.
Swanson KS, Dowd SE, Suchodolski JS*, et al.* (2011) Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *ISME J* **5**: 639-649.

Tan Z, Wang Y, Yang T, Ao H, Chen S, Xing K, Zhang F, Zhao X, Liu J & Wang C (2018) Differences in gut microbiota composition in finishing Landrace pigs with low and high feed conversion ratios. *Antonie Van Leeuwenhoek* **111**: 1673-1685.
Tandina F, Almeras L, Kone AK, Doumbo OK, Raoult D & Parola P (2016) Use of MALDI-TOF MS and culturomics to identify mosquitoes and their midgut microbiota. *Parasit Vectors* **9**: 495.
Tap J, Mondot S, Levenez F*, et al.* (2009) Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol* **11**: 2574-2584.
Thursby E & Juge N (2017) Introduction to the human gut microbiota. *Biochem J* **474**: 1823-1836.
Tissier H (1900) *Recherches sur la flore intestinale des nourrissons: état normal et pathologique*.
Tomasello G, Mazzola M, Leone A*, et al.* (2016) Nutrition, oxidative stress and intestinal dysbiosis: Influence of diet on gut microbiota in inflammatory bowel diseases. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **160**: 461-466.
Trovatelli LD, Crociani F, Pedinotti M & Scardovi V (1974) Bifidobacterium pullorum sp. nov.: a new species isolated from chicken feces and a related group of bifidobacteria isolated from rabbit feces. *Arch Microbiol* **98**: 187-198.
Tun HM, Brar MS, Khin N, Jun L, Hui RK, Dowd SE & Leung FC (2012) Gene-centric metagenomics analysis of feline intestinal microbiome using 454 junior pyrosequencing. *J Microbiol Methods* **88**: 369-376.
Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R & Gordon JI (2007) The human microbiome project. *Nature* **449**: 804-810.
Turroni F, van Sinderen D & Ventura M (2009) Bifidobacteria: from ecology to genomics. *Front Biosci (Landmark Ed)* **14**: 4673-4684.
Turroni F, van Sinderen D & Ventura M (2011) Genomics and ecological overview of the genus Bifidobacterium. *Int J Food Microbiol* **149**: 37-44.
Turroni F, Milani C, van Sinderen D & Ventura M (2011) Genetic strategies for mucin metabolism in Bifidobacterium bifidum PRL2010: an example of possible human-microbe co-evolution. *Gut Microbes* **2**: 183-189.
Turroni F, Ribbera A, Foroni E, van Sinderen D & Ventura M (2008) Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek* **94**: 35-50.
Turroni F, Milani C, Duranti S, Mahony J, van Sinderen D & Ventura M (2018) Glycan Utilization and Cross-Feeding Activities by Bifidobacteria. *Trends Microbiol* **26**: 339-350.
Turroni F, Milani C, Duranti S, Ferrario C, Lugli GA, Mancabelli L, van Sinderen D & Ventura M (2018) Bifidobacteria and the infant gut: an example of co-evolution and natural selection. *Cell Mol Life Sci* **75**: 103-118.
Turroni F, Foroni E, O'Connell Motherway M*, et al.* (2010) Characterization of the serpin-encoding gene of Bifidobacterium breve 210B. *Appl Environ Microbiol* **76**: 3206-3219.
Turroni F, Foroni E, Pizzetti P*, et al.* (2009) Exploring the diversity of the bifidobacterial population in the human intestinal tract. *Appl Environ Microbiol* **75**: 1534-1545.
Turroni F, Milani C, Duranti S*, et al.* (2016) Deciphering bifidobacterial-mediated metabolic interactions and their impact on gut microbiota by a multi-omics approach. *ISME J* **10**: 1656-1668.
Turroni F, Peano C, Pass DA*, et al.* (2012) Diversity of bifidobacteria within the infant gut microbiota. *PLoS One* **7**: e36957.
Turroni F, Bottacini F, Foroni E*, et al.* (2010) Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc Natl Acad Sci U S A* **107**: 19514-19519.
Turroni F, Serafini F, Foroni E*, et al.* (2013) Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions. *Proc Natl Acad Sci U S A* **110**: 11151-11156.
Ushida K, Tsuchida S, Ogura Y, Toyoda A & Maruyama F (2016) Domestication and cereal feeding developed domestic pig-type intestinal microbiota in animals of suidae. *Anim Sci J* **87**: 835-841.

Vandeputte D, Kathagen G, D'Hoe K*, et al.* (2017) Quantitative microbiome profiling links gut community variation to microbial load. *Nature* **551**: 507-511.
Vazquez-Baeza Y, Hyde ER, Suchodolski JS & Knight R (2016) Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. *Nat Microbiol* **1**: 16177.
Ventura M, Turroni F, Canchaya C, Vaughan EE, O'Toole PW & van Sinderen D (2009) Microbial diversity in the human intestine and novel insights from metagenomics. *Front Biosci (Landmark Ed)* **14**: 3214-3221.
Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF & van Sinderen D (2007) Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* **71**: 495-548.
Verberkmoes NC, Russell AL, Shah M*, et al.* (2009) Shotgun metaproteomics of the human distal gut microbiota. *ISME J* **3**: 179-189.
Vieira AT, Teixeira MM & Martins FS (2013) The role of probiotics and prebiotics in inducing gut immunity. *Front Immunol* **4**: 445.
Vital M, Gao J, Rizzo M, Harrison T & Tiedje JM (2015) Diet is a major factor governing the fecal butyrate-producing community structure across Mammalia, Aves and Reptilia. *ISME J* **9**: 832-843.
Wanapat M, Cherdthong A, Phesatcha K & Kang S (2015) Dietary sources and their effects on animal production and environmental sustainability. *Anim Nutr* **1**: 96-103.
Wang WL, Xu SY, Ren ZG, Tao L, Jiang JW & Zheng SS (2015) Application of metagenomics in the human gut microbiome. *World J Gastroenterol* **21**: 803-814.
Wang Z, Bergeron N, Levison BS*, et al.* (2019) Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. *Eur Heart J* **40**: 583-594.
Watabe Junko BY, Mitsuoka Tomotari (1983) *Bifidobacterium gallinarum* sp. nov.: a new species isolated from the ceca of chickens. *International Journal of Systematic Bacteriology* **33**: 127-132.
Watanabe K, Makino H, Sasamoto M, Kudo Y, Fujimoto J & Demberel S (2009) Bifidobacterium mongoliense sp. nov., from airag, a traditional fermented mare's milk product from Mongolia. *Int J Syst Evol Microbiol* **59**: 1535-1540.
Wu GD, Chen J, Hoffmann C*, et al.* (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**: 105-108.
Wu X, Zhang H, Chen J, Shang S, Yan J, Chen Y, Tang X & Zhang H (2017) Analysis and comparison of the wolf microbiome under different environmental factors using three different data of Next Generation Sequencing. *Sci Rep* **7**: 11332.
Xu J, Verbrugghe A, Lourenco M*, et al.* (2016) Does canine inflammatory bowel disease influence gut microbial profile and host metabolism? *BMC Vet Res* **12**: 114.
Zhao Y, Tang H & Ye Y (2012) RAPSearch2: a fast and memory-efficient protein similarity search tool for next-generation sequencing data. *Bioinformatics* **28**: 125-126.
Zoetendal EG, Rajilic-Stojanovic M & de Vos WM (2008) High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* **57**: 1605-1615.
Zomer A, Fernandez M, Kearney B, Fitzgerald GF, Ventura M & van Sinderen D (2009) An interactive regulatory network controls stress response in Bifidobacterium breve UCC2003. *J Bacteriol* **191**: 7039-7049.

Publications

1. Milani C*, **Alessandri G***, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Longhi G, Anzalone R, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Deciphering the impact of diet and host physiology on the mammalian gut microbiome by multi-omics approaches.**" Appl Environ Microbiol. 2020 Sep 18:AEM.01864-20. doi: 10.1128/AEM.01864-20. *These author contributed equally.
2. Milani C, **Alessandri G**, Mangifesta M, Mancabelli L, **Lugli GA**, Fontana F, Longhi G, Anzalone R, Viappiani A, Duranti S, Turroni F, Costi R, Annicchiarico A, Morini A, Sarli L, Ossiprandi MC, van Sinderen D, Ventura M. "**Untangling Species-Level Composition of Complex Bacterial Communities through a Novel Metagenomic Approach.**" mSystems. 2020 Jul 28;5(4):e00404-20. doi: 10.1128/mSystems.00404-20.
3. Lugli GA, **Alessandri G**, Milani C, Mancabelli L, Ruiz L, Fontana F, Borragán S, González A, Turroni F, Ossiprandi MC, Margolles A, van Sinderen D, Ventura M. "**Evolutionary Development and Co-Phylogeny of Primate-Associated Bifidobacteria.**" Environ Microbiol. 2020 Aug;22(8):3375-3393. doi: 10.1111/1462-2920.15108.
4. Milani C, Fontana F, **Alessandri G**, Mancabelli L, Lugli GA, Longhi G, Anzalone R, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Ecology of lactobacilli present in Italian cheeses produced from raw milk.**" Appl Environ Microbiol. 2020 Jun 2;86(12):e00139-20. doi: 10.1128/AEM.00139-20.
5. Lugli GA, Duranti S, Milani C, Mancabelli L, Turroni F, **Alessandri G**, Longhi G, Anzalone R, Viappinai A, Tarrachini C, Bernasconi S, Yonemitsu C, Bode L, Goran MI, Ossiprandi MC, van Sinderen D, Ventura M. "**Investigating bifidobacteria and human milk oligosaccharide composition of lactating mothers.**" FEMS Microbiol Ecol. 2020 May 1;96(5):fiaa049. doi: 10.1093/femsec/fiaa049.
6. **Alessandri G**, Argentini C, Milani C, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M- **"Catching a glimpse of the gut community of companion animals: a canine and feline perspective".** Microbi Biotechnol. 2020 Nov;13(6):1708-1732. doi: 10.1111/1751-7915.13656.
7. Duranti S, Lugli GA, Viappiani A, Mancabelli L, **Alessandri G**, Anzalone R, Longhi G, Milani C, Ossiprandi MC, Turroni F, Ventura M. "**Characterization of the phylogenetic diversity of two novel species belonging to the genus *Bifidobacterium*: *Bifidobacterium cebidarum* sp. nov. and *Bifidobacterium leontopitheci* sp. nov.**" Int J Syst Evol Microbiol. 2020 Apr;70(4):2288-2297. doi: 10.1099/ijsem.0.004032.
8. **Alessandri G**, Milani C, Mancabelli L, Longhi G, Anzalone R, Lugli GA, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Deciphering the bifidobacterial population within the canine and feline gut microbiota.**" Appl Environ Microbiol. 2020 Mar 18;86(7). pii: e02875-19. doi: 10.1128/AEM.02875-19.
9. Milani C, **Alessandri G**, Mancabelli L, Lugli GA, Longhi G, Anzalone R, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Bifidobacterial Distribution Across Italian Cheeses Produced from Raw Milk.**" Microorganisms. 2019 Nov 21;7(12). pii: E599. doi: 10.3390/microorganisms7120599.
10. **Alessandri G***, Milani C*, Duranti S*, Mancabelli L, Ranjanoro T, Modica S, Carnevali L, Statello R, Bottacini F, Turroni F, Ossiprandi MC, Sgoifo A, van Sinderen D, Ventura M. "**Ability of bifidobacteria to metabolize chitin-glucan and its impact on the gut microbiota"**. Sci Rep. 2019 Apr 8;9(1):5755. doi: 10.1038/s41598-019-42257-z. *These author contributed equally.

11. Milani C, Duranti S, Napoli S, **Alessandri G**, Mancabelli L, Anzalone R, Longhi G, Viappiani A, Mangifesta M, Lugli GA, Bernasconi S, Ossiprandi MC, van Sinderen D, Ventura M, Turroni F. "**Colonization of the human gut by bovine bacteria present in Parmesan cheese.**" Nat Commun. 2019 Mar 20;10(1):1286. doi: 10.1038/s41467-019-09303-w.
12. **Alessandri G**, Ossiprandi MC, MacSharry J, van Sinderen D, Ventura M. "**Bifidobacterial dialogue with its human host and consequent modulation of the immune system**". Front Immunol. 2019 Oct 1;10:2348. doi: 10.3389/fimmu.2019.02348.
13. Duranti S, Lugli GA, Milani C, James K, Mancabelli L, Turroni F, **Alessandri G**, Mangifesta M, Mancino W, Ossiprandi MC, Iori A, Rota C, Gargano G, Bernasconi S, Di Pierro F, van Sinderen D, Ventura M. "***Bifidobacterium bifidum* and the infant gut microbiota: an intriguing case of microbe-host co-evolution.**" Environ Microbiol. 2019 Oct;21(10):3683-3695. doi: 10.1111/1462-2920.14705.
14. **Alessandri G**, Milani C, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**The impact of human-facilitated selection on the gut microbiota of domesticated mammals.**" FEMS Microbiol Ecol. 2019 Sep 1;95(9). pii: fiz121. doi: 10.1093/femsec/fiz121.
15. Lugli GA, Milani C, Duranti S, **Alessandri G**, Turroni F, Mancabelli L, Tatoni D, Ossiprandi MC, van Sinderen D, Ventura M. "**Isolation of novel gut bifidobacteria using a combination of metagenomic and cultivation approaches.**" Genome Biol. 2019 May 16;20(1):96. doi: 10.1186/s13059-019-1711-6.
16. Duranti S, Lugli GA, Napoli S, Anzalone R, Milani C, Mancabelli L, **Alessandri G**, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Characterization of the phylogenetic diversity of five novel species belonging to the genus *Bifidobacterium*: *Bifidobacterium castoris* sp. nov., *Bifidobacterium callimiconis* sp. nov., *Bifidobacterium goeldii* sp. nov., *Bifidobacterium samirii* sp. nov. and *Bifidobacterium dolichotidis* sp. nov.**" Int J Syst Evol Microbiol. 2019 May;69(5):1288-1298. doi: 10.1099/ijsem.0.003306.
17. Lugli GA, Duranti S, Albert K, Mancabelli L, Napoli S, Viappiani A, Anzalone R, Longhi G, Milani C, Turroni F, **Alessandri G**, Sela DA, van Sinderen D, Ventura M. "**Unveiling genomic diversity among members of the *Bifidobacterium pseudolongum* species, a widely distributed gut commensal of the animal kingdom.**" Appl Environ Microbiol. 2019 Apr 4;85(8). pii: e03065-18. doi: 10.1128/AEM.03065-18.
18. **Alessandri G***, Milani C*, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features.**" Environ Microbiol. 2019 Apr;21(4):1331-1343. doi: 10.1111/1462-2920.14540.
19. Milani C, Duranti S, Mangifesta M, Lugli GA, Turroni F, Mancabelli L, Viappiani A, Anzalone R, **Alessandri G**, Ossiprandi MC, van Sinderen D, Ventura M. "**Phylotype-Level Profiling of Lactobacilli in Highly Complex Environments by Means of an Internal Transcribed Spacer-Based Metagenomic Approach.**" Appl Environ Microbiol. 2018 Jul 2;84(14). pii: e00706-18. doi: 10.1128/AEM.00706-18.
20. Lugli GA, Mangifesta M, Duranti S, Anzalone R, Milani C, Mancabelli L, **Alessandri G**, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. "**Phylogenetic classification of six novel species belonging to the genus *Bifidobacterium* comprising *Bifidobacterium anseris* sp. nov., *Bifidobacterium criceti* sp. nov., *Bifidobacterium imperatoris* sp. nov., *Bifidobacterium italicum* sp. nov., *Bifidobacterium margollesii* sp. nov. and *Bifidobacterium parmae* sp. nov.**" Syst Appl Microbiol. 2018 May;41(3):173-183. doi: 10.1016/j.syapm.2018.01.002.