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Multi-omics Approaches To Decipher the Impact of Diet and Host Physiology on the Mammalian Gut Microbiome

Christian Milani,[b,d] Giulia Alessandri,[a] Leonardo Mancabelli,[b] Marta Mangifesta,[b] Gabriele Andrea Lugli,[b] Alice Viappiani,[c] Giulia Longhi,[c] Rosaria Anzalone,[c] Sabrina Duranti,[b] Francesca Turroni,[b,d] Maria Cristina Ossiprandi,[a,d] Douwe van Sinderen,[e] Marco Ventura[b,d]

[a]Department of Veterinary Medical Science, University of Parma, Parma, Italy
[b]Laboratory of Probiogenomics, Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parma, Italy
[c]GenProbio srl, Parma, Italy
[d]Microbiome Research Hub, University of Parma, Parma, Italy
[e]APC Microbiome Institute and School of Microbiology, National University of Ireland, Cork, Ireland

Christian Milani and Giulia Alessandri contributed equally to this article. Author order is on the basis of seniority.

ABSTRACT In recent years, various studies have demonstrated that the gut microbiota influences host metabolism. However, these studies were focused primarily on a single or a limited range of host species, thus preventing a full exploration of possible taxonomic and functional adaptations by gut microbiota members as a result of host-microbe coevolution events. In the current study, the microbial taxonomic profiles of 250 fecal samples, corresponding to 77 host species that cover the mammalian branch of the tree of life, were reconstructed by 16S rRNA gene-based sequence analysis. Moreover, shotgun metagenomics was employed to investigate the metabolic potential of the fecal microbiomes of 24 mammals, and subsequent statistical analyses were performed to assess the impact of host diet and corresponding physiology of the digestive system on gut microbiota composition and functionality. Functional data were confirmed and extended through metatranscriptome assessment of gut microbial populations of eight animals, thus providing insights into the transcriptional response of gut microbiota to specific dietary lifestyles. Therefore, the analyses performed in this study support the notion that the metabolic features of the mammalian gut microbiota have adapted to maximize energy extraction from the host's diet.

IMPORTANCE Diet and host physiology have been recognized as main factors affecting both taxonomic composition and functional features of the mammalian gut microbiota. However, very few studies have investigated the bacterial biodiversity of mammals by using large sample numbers that correspond to multiple mammalian species, thus resulting in an incomplete understanding of the functional aspects of their microbiome. Therefore, we investigated the bacterial taxonomic composition of 250 fecal samples belonging to 77 host species distributed along the tree of life in order to assess how diet and host physiology impact the intestinal microbial community by selecting specific microbial players. Conversely, the application of shotgun metagenomics and metatranscriptomics approaches to a group of selected fecal samples allowed us to shed light on both metabolic features and transcriptional responses of the intestinal bacterial community based on different diets.

KEYWORDS microbiota, metagenomics, mammals, metatranscriptomics, diet, physiology

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Address correspondence to Marco Ventura, marco.ventura@unipr.it.

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The functional roles exerted by the mammalian gut microbiota have in recent years been scrutinized by a range of studies focusing on multiple aspects of host biology, including the immune, digestive, and nervous systems (1–4). In this regard, gut microbiota composition has been shown to be influenced by host genetics (5–11) as well as environmental factors that are linked to host lifestyle and diet (7, 10, 12–14). Microbe-host interactions are the result of intricate adaptive occurrences, through a process known as host-microbe coevolution, being responsible for the adaptation of mammals to new environmental niches and having contributed to their dispersal and current global distribution (5, 15). Among multiple factors, diet, host evolutionary history, and host physiology are currently presumed to be the main drivers implicated in the modulation of the mammalian gut microbiota (5, 7, 12, 13, 16–18). In this context, several comparative analyses of mammalian gut microbial communities have revealed associations between the composition of the gut ecosystem and host diet, even among phylogenetically unrelated hosts (5, 18), and supported the notion that diet contributes to the microbiome plasticity by selecting particular metabolic activities to allow degradation of specific components of the host diet (5, 18, 19). Specifically, while carnivorous communities have been reported to be specialized in the degradation of proteins, herbivorous microbiomes harbor genes that encode enzymatic activities involved in the breakdown of complex plant-derived polysaccharides, that are absent in the genetic repertoire of their host, and that synthesize amino acid building blocks to cope with protein deficiency typical of their diet (18, 20, 21).

In concert with diet, host phylogeny and physiology have been proposed as crucial factors affecting the mammalian gut microbial community (5, 10, 16, 17). In recent years, the term "phylosymbiosis" has been proposed to define the eco-evolutionary pattern that associates host evolutionary changes with ecological modulations of their intestinal microbial community (22, 23). Indeed, despite the interindividual fluctuations of gut microbiomes and the possible rapid changes in response to diet and environment, it has been demonstrated that the mammalian gut microbiota composition diverges at a relatively constant rate across an evolutionary timescale (10, 24), suggesting that host traits that undergo changes across host phylogeny, including gut physiology, have an important role in shaping the intestinal microbial community across mammals (7). However, this conserved pattern of host-microbe phylosymbiosis seems to be restricted to mammals. Indeed, meta-analyses performed on fecal samples of various bird, fish, reptile, or amphibian species failed to report the same strict correlation (7, 16). Together, these findings indicate that the gut microbiota plays a pivotal role in facilitating adaptation to dietary changes adopted by mammals as part of their evolution, revealing particular correlations between a given gut microbiota and its associated host diet and/or digestive system (5, 7, 9, 10).

Nevertheless, despite many studies depicting the gut microbiota as a hidden organ that exerts key metabolic activities to support its host, the composition and especially the functional role of mammalian gut microbial populations have not been fully explored. Indeed, despite the extensive number of mammalian species involved, most of the available studies explored the mammalian gut microbiota composition exclusively through 16S rRNA microbial profiling, thus failing to provide a correlation between the composition of the mammalian gut microbiota and its (predicted) metabolic functions (5, 7, 10, 16, 25). Other studies, even though they were based on shotgun metagenomics, did not investigate transcriptional profiles of the collected samples. In this context, in order to expand our knowledge in this field, the specific taxonomic and functional traits associated with different diets and the physiology of the host's digestive system across the mammalian branch of the tree of life were assessed by means of metagenomics (16S rRNA microbial profiling and shotgun metagenomics) and metatranscriptomics approaches. Specifically, we collected fecal samples from 250 mammals, covering 77 species and representing a broad range of mammalian biodiversity. These samples were subjected to 16S rRNA gene microbial profiling in order to obtain an overview of the taxonomic composition of the gut microbiota among their mammalian hosts. Moreover, 24 key samples were subjected to

shotgun metagenomic sequencing and reconstruction of their microbial metabolic potential in order to identify features that allow adaptation to specific diets linked with various evolved physiologies of the mammalian gastrointestinal tract. These functional data were confirmed and integrated by data obtained by metatranscriptome analysis of eight animals, thereby providing insights into the transcriptional response of gut microbiota populations to specific diets.

RESULTS AND DISCUSSION

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 (see Table S1 in the 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 nondomesticated 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.

Evaluation of the alpha diversity, i.e., the biodiversity of the bacterial population harbored 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 than that of other mammals, supported by Student's *t* test *P* value of <0.001 (Fig. 1a). 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 than that of omnivores or carnivores (the latter including piscivores) (Fig. S1a). These data indicate that the overall bacterial biodiversity harbored 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 Fig. S1b). The average rarefaction curves that we obtained revealed that polygastric herbivores, including ruminants and pseudoruminants (Tylopoda), possess a significantly higher gut microbiota biodiversity (Fig. S1b), reflecting the key role of foregut bacterial fermentation in herbivores with a multichambered stomach (26). 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 nonruminant herbivores (26). In contrast, herbivores with a single-chambered stomach showed significant variation in the number of observed OTUs based on their size (Fig. S1b). In detail, "lighter" ($<$100 kg average body weight) monogastric herbivores (representing five mammalian species and an associated total of 18 fecal samples) were shown to exhibit lower biodiversity than that of "heavier" ($>$100 kg 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 (26). 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

TABLE 1 Mammals whose fecal samples were collected for this study[a]

Diet group	Common name	Species	Family	No. of sampled individuals
Herbivore (polygastric ruminant)	African mouflon	*Ammotragus lervia*	Bovidae	2
	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	*Giraffa camelopardalis*	Giraffidae	2
Herbivore (polygastric Tylopoda)	Camel	*Camelus bactrianus*	Camelidae	2
	Llama	*Lama glama*	Camelidae	1
	Guanaco	*Lama guanicoe*	Camelidae	3
	Alpaca	*Vicugna pacos*	Camelidae	7
	Vicuna	*Vicugna vicugna*	Camelidae	1
Herbivore (polygastric nonruminant, 3 stomachs)	Pygmy hippopotamus	*Hexaprotodon liberiensis*	Hippopotamidae	5
	Hippopotamus	*Hippopotamus amphibius*	Hippopotamidae	3
	Grey kangaroo	*Macropus giganteus*	Macropodidae	1
Herbivore (monogastric, <100 kg)	Hare	*Lepus europaeus*	Leporidae	9
	European rabbit	*Oryctolagus cuniculus*	Leporidae	4
	European beaver	*Castor fiber*	Castoridae	2
	Patagonian mara	*Dolichotis patagonum*	Caviidae	1
	Capybara	*Hydrochoerus hydrochaeris*	Caviidae	2
Herbivore (monogastric, >100 kg)	African wild donkey	*Equus africanus*	Equidae	4
	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
Carnivore	Wolf	*Canis lupus*	Canidae	10
	Dog	*Canis lupus familiaris*	Canidae	25
	African wild dog	*Lycaon pictus*	Canidae	1
	Wildcat	*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
Omnivore	Wild boar	*Sus scrofa*	Suidae	8
	Pig	*Sus scrofa domesticus*	Suidae	10
	Pygmy marmoset	*Callithrix pygmaea*	Cebidae	1

(Continued on next page)

TABLE 1 (Continued)

Diet group	Common name	Species	Family	No. of sampled individuals
	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 mangabey	*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
Piscivore	Dolphin	*Delphinus delphis*	Delphinidae	3
	Bottlenose dolphin	*Tursiops truncatus*	Delphinidae	3

[a]The number of sampled individuals per mammalian species and their diets are included.

the encumbrance of a large hindgut, thus being advantageous for small animals with a high ratio of food intake to size (26). In contrast, in colon fermenters, the contents of colon and cecum mix freely and the colon and cecum act as a single fermentation site (26), possibly supporting the higher bacterial biodiversity observed in heavier monogastric herbivores (Fig. 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 (Fig. 1b; Fig. S2a). This analysis revealed clustering of samples based on taxonomic family, as expected, with overlap of families with similar diets (Fig. S2a). In fact, recoloring of the samples based on dietary habits revealed that herbivores, omnivores, and carnivores (including piscivores) clustered separately (Fig. 1b), with herbivores forming subclusters, confirming previously published observations (18). In order to detail differences between herbivores, a specific Bray-Curtis PCoA was generated (Fig. S2b). The latter revealed three major clusters constituted by (i) polygastric ruminants and pseudoruminants (Tylopoda), (ii) heavier monogastric herbivores, and (iii) lighter monogastric herbivores and Hippopotamidae (Fig. S2b). These findings highlight that diet, as well as 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 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 (Fig. 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 (Fig. 2b). In this context, it has previously been shown that the *Fusobacterium* genus is generally associated with a protein-rich diet (27), while a high abundance of members of the *Ruminococcaceae* family is related to a fiber-based diet, since the latter are degraders of a wide range of carbohydrates (28). Nevertheless, although our findings indicate that members of these two bacterial taxonomic groups

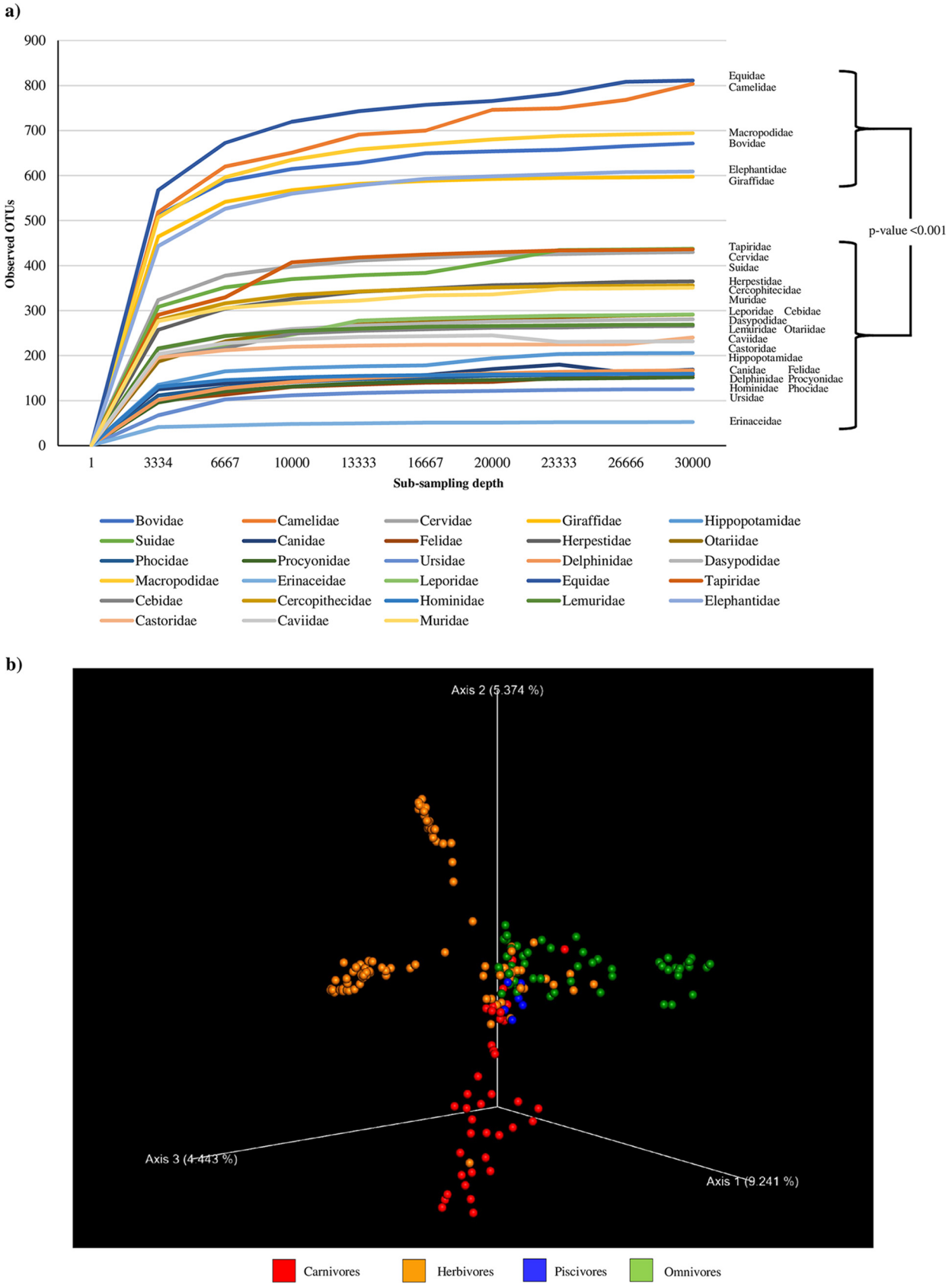
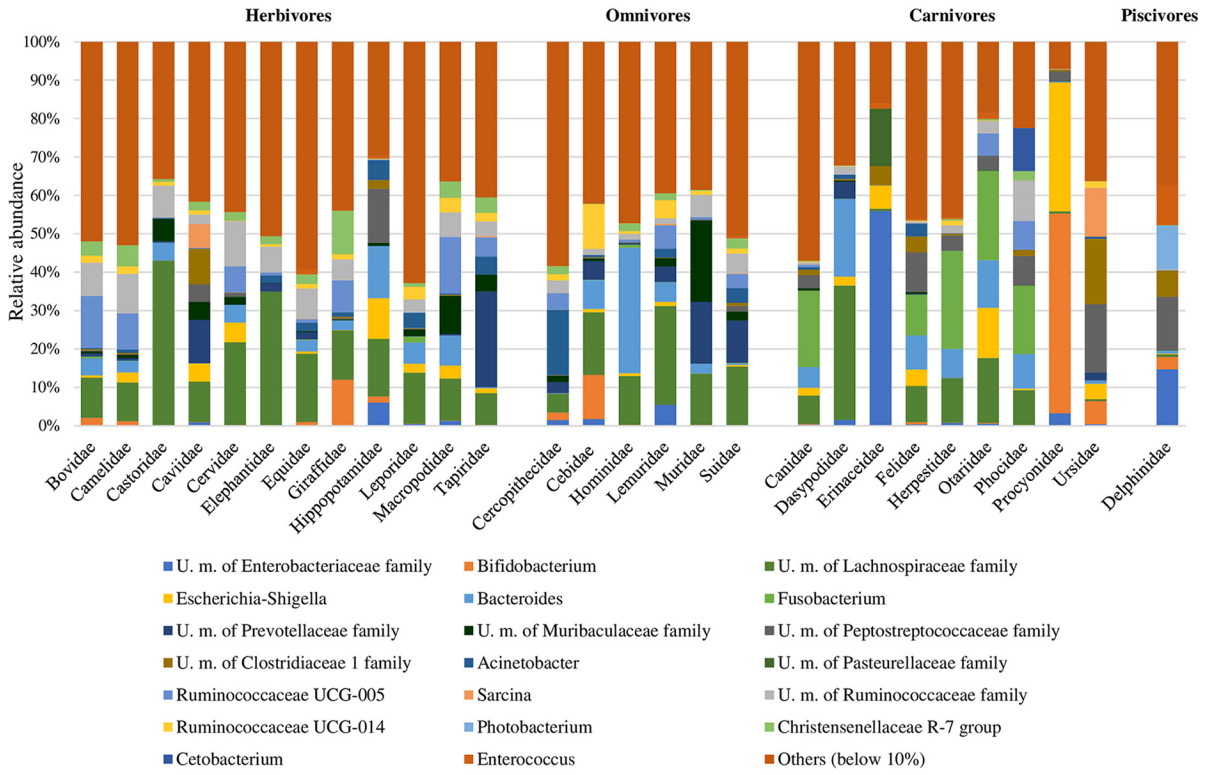


FIG 1 Alpha and beta diversity of mammals included in this study. (a) Average rarefaction curves obtained for each mammalian taxonomic family through evaluation of the number of observed OTUs up to 30,000 reads. (b) PCoA representation obtained using the Bray-Curtis index and genus-level profiles. Samples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

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

FIG 2 Impact of diet on mammalian gut microbiota genus-level taxonomic composition. (a) Bar plot of the average genus-level taxonomic composition obtained for each mammalian taxonomic family. Taxonomic families are grouped by diet. U. m., unclassified member. (b) Bacterial genera with average relative abundances two times higher in mammals following a specific diet than in mammals following the other three considered diets. The abundances of these taxa are highlighted in green.

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 (Fig. 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 (Fig. S3b), suggesting that the peculiar gut microbiota composition of cecum fermenters may reflect their shorter transit time and specific energy extraction capabilities in comparison to colon fermenters, i.e., heavier monogastric animals (>100 kg average body weight), and ruminants (26).

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 (29). In this context, previous studies have revealed the existence of both cooperative and competitive behaviors between members of the mammalian gut microbiota (29–31). 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 (Fig. 3a). In this context, coloring of the nodes based on modularity class analysis (resolution of 0.6) revealed the presence of three major clusters organized 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 (Fig. 3a). Moreover, node coloring corresponding to taxa found to be associated with specific diets (*P* value < 0.05) (Fig. 2 and 3b) revealed that genera more abundant in herbivores, carnivores, and piscivores clustered together, thus suggesting the existence of putative cooperational behaviors 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 (25). 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; Fig. 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 (Fig. 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 than of those corresponding to carnivores (Fig. 3c).

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

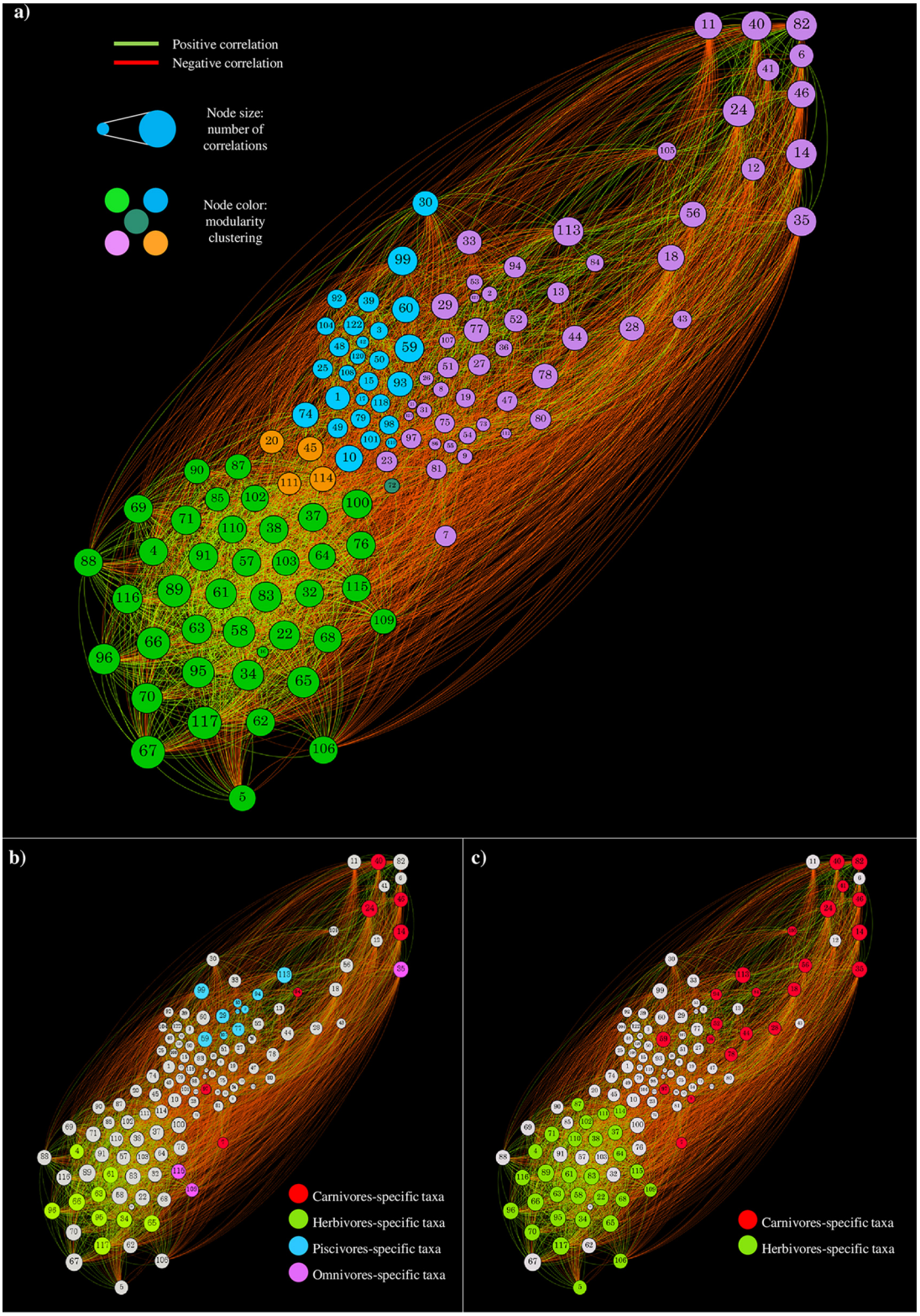


FIG 3 Covariance force-driven network of genera profiled with a relative abundance of >5% in at least one sample. Nodes represent genera included in the analysis, and attraction and repulsion forces are proportional to statistically significant covariances and coexclusions obtained using Kendall's tau correlation coefficient. Node size is proportional to the number of correlations. (a) Network with nodes colored based on the predicted modularity class (using 0.6 resolution). (b and c) The same network as shown in panel a but with nodes colored to highlight bacterial genera identified as more abundant in a specific diet through analysis of carnivores, herbivores, piscivores, and omnivores (b) as well as between only carnivores and herbivores (c).

(Continued on next page)

(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 data sets 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 data sets) and a higher number of pathways with lower abundance than the gut microbiota of animals on both other diets (Fig. 4), thus allowing formulation of the hypothesis that aquatic life and correlated diet induced an 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) (Fig. 4). In-depth evaluation of degradative pathways showing higher abundance for a specific diet (Bonferroni *post hoc* test P value < 0.05) (Fig. 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 (32–34) (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 superpathway of trimethylamine degradation (Table S5). Notably, choline, a quaternary amine principally found in meats, is known as a precursor of trimethylamine (35, 36). 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 that suggested that the carnivore microbiome is specialized to derive energy from protein degradation (18). Collectively, these findings support the notion that diet plays a role in modulating the taxonomic composition of the intestinal microbial

FIG 3 Legend (Continued)
Genera are indicated by number as follows: *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 coprostanoligenes* 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), unclassified member (U. m.) of *Rickettsiales* 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 *Peptostreptococcaceae* 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).

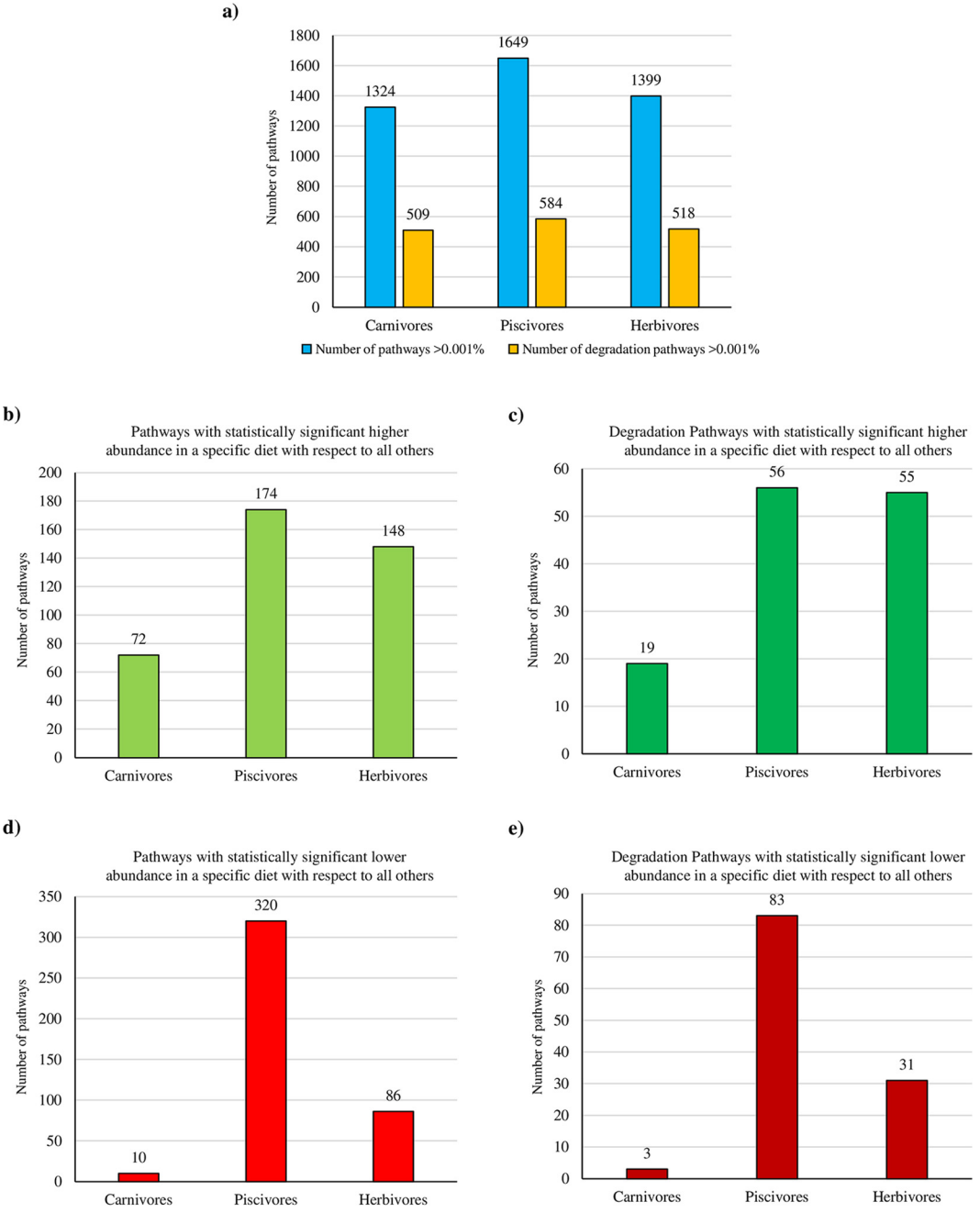


FIG 4 Metabolic pathway prediction in carnivores, piscivores, and herbivores. (a) Number of pathways detected with an abundance of >0.001%. (b and c) Sums of the numbers of all pathways (b) and degradative pathways (c) that showed a significantly higher abundance in a specific diet than in the other two considered diets observed through the application of an ANOVA *post hoc* Bonferroni statistical analysis. (d and e) Sums of the numbers of all pathways (d) and degradative pathways (e) with a significantly lower abundance in a specific diet than in the other two.

community, with a consequent impact of the metabolic pathways encoded by these mammalian intestinal microbial communities.

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 the 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 statistical analysis

TABLE 2 GH families with statistically significant higher or lower abundance based on diet

GH family	Abundance (%)[a] in:		
	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

[a]Percentages in bold indicate significance at a Bonferroni *post hoc* test *P* value of <0.05 in comparison to other groups.

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 $\alpha(1\rightarrow4)$-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.

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 toward the 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 (Tables S5 and S6; Fig. 5).

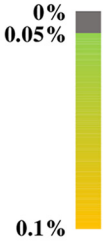
Inspection of transcriptional data revealed that the range of pathways involved in the breakdown of typical plant carbohydrates, i.e., xylose, arabinose, and starch, found to be more abundant in herbivores based on shotgun metagenomic data (Table S5), are also more expressed in animals that follow this 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.

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 (Fig. 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 (Fig. 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 (Fig. 5a), despite a comparable abundance of their corresponding genes in metagenomic data sets of carnivores. In addition, the superpathway of methanogenesis showed higher expression in herbivores (Fig. 5b), possibly reflecting the major metabolic role exerted by methanogens in this class of mammals (37).

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 in order 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 pseudoruminants (Tylopoda), heavier monogastric herbivores ($>$100 kg average body weight), and lighter monogastric herbivores ($<$100 kg average body weight). Notably, comparison of the gut microbiomes of polygastric ruminants and pseudoruminants revealed very limited differences in terms of encoded pathways and predicted glycobiome (Tables S8 and S9). In detail, only one metabolic pathway with a relative abundance of $>$0.001% was found to show increased abundance in ruminants $\pm$50% compared to pseudoruminants (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 similar multichambered digestive systems may exert comparable metabolic functions (26, 38). Indeed, comparison of the numbers of pathways with a

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

FIG 5 Metatranscriptome profiles of carnivores and herbivores. (a) Transcriptional abundance (as a proportion of the total glycobiome) of GH genes with statistically different abundances in carnivores and herbivores. GHs in red showed similar abundances in the metagenomes of carnivores and herbivores. (b) Transcriptional abundance (as a proportion of all predicted metabolic pathways) of degradation pathways with statistically different abundances in carnivores and herbivores. Pathways in red displayed similar abundances in the metagenomes of carnivores and herbivores.

statistically significant differential abundance between the two groups of monogastric herbivores and ruminants or pseudoruminants revealed similar trends, with the only exception being a slight decrease in the number of pathways with statistically significant higher abundance in the pseudoruminants than in monogastric herbivores (Table S10). For this reason, ruminants and pseudoruminants were considered 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% are lower in polygastric animals than in monogastric herbivores.

Furthermore, our collected data revealed that the gut microbiota of ruminants and pseudoruminants encodes the highest number of pathways with statistically significant lower abundance in comparison to monogastric herbivores (Fig. 6), with a similar trend observed for degradative pathways (Fig. 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.

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 (Fig. 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) (Fig. S1b), probably reflecting the limited colon size responsible for their specialization as cecum fermenters (26). On the basis of these two observations, it can be assumed that the intestinal bacterial community of lighter monogastric mammals compensates for 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 (39–41) (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 sulfate into hydrogen sulfide (Table S10). This observation may suggest that the higher biodiversity of heavier monogastric herbivores (Fig. 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 (Fig. 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 disaccharides) 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 either to represent chitinase activity (associated with GH19), which participates in the hydrolysis of (1→4) β-linkages between *N*-acetyl-

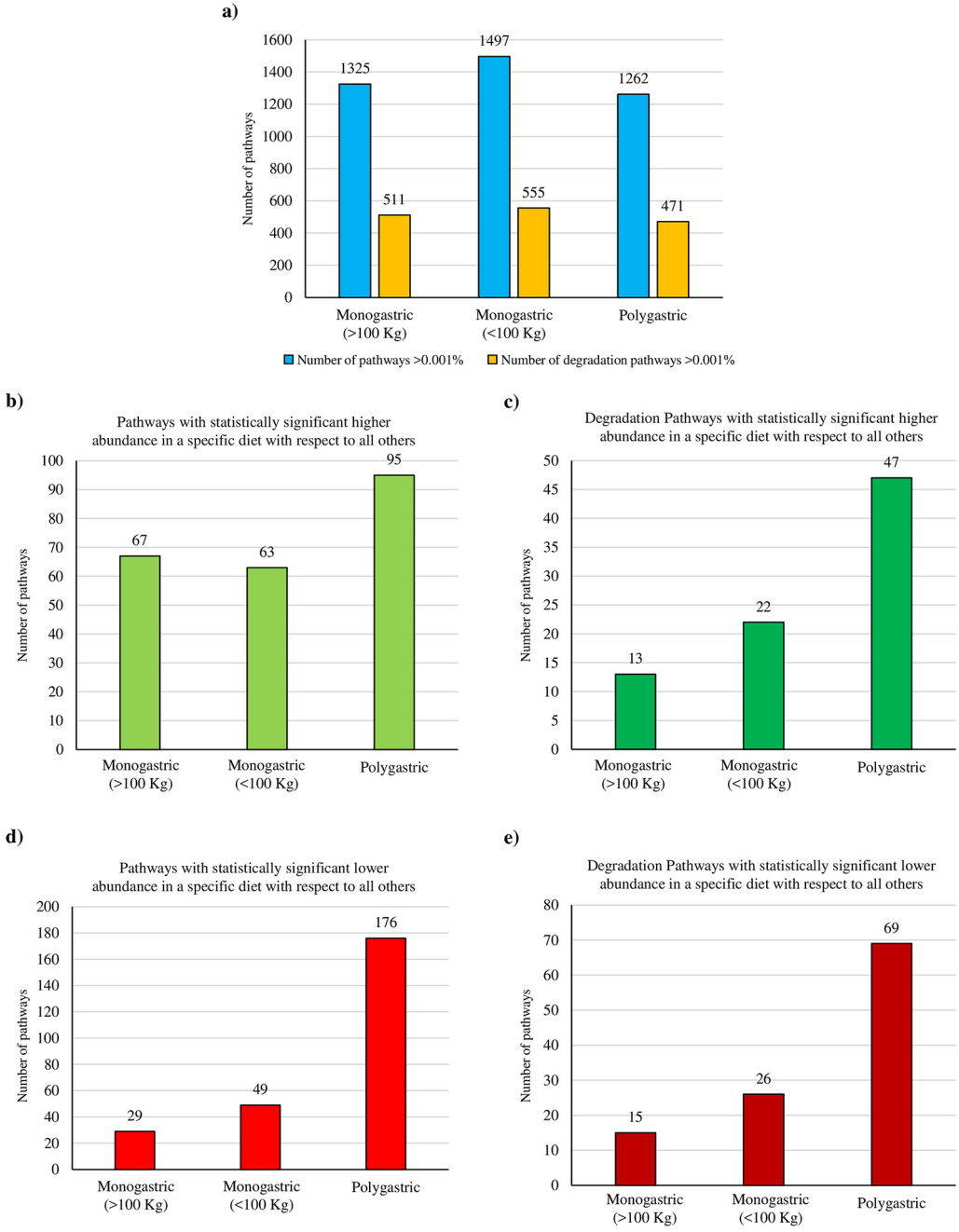


FIG 6 Metabolic pathway prediction in lighter monogastric, heavier monogastric, and polygastric herbivores. (a) Sums of the number of pathways detected with an abundance of >0.001%. (b and c) Sums of the numbers of all pathways (b) and degradative pathways (c) with significantly higher abundance in a specific class of herbivores. (d and e) Sums of the numbers of all pathways (d) and degradative pathways (e) with significantly lower abundance in a specific class of herbivores. Statistically significant differences were defined by applying ANOVA *post hoc* Bonferroni statistical analysis.

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 (42) (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

TABLE 3 GH families with statistically significant higher or lower abundance based on digestive system's physiology

GH family	Abundance (%)[a] in:		
	Heavier monogastric herbivores	Lighter monogastric herbivores	Polygastric herbivores
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

[a]Percentages in bold indicate significance at a Bonferroni *post hoc* test *P* value of <0.05 in comparison to other groups.

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 3,981%) in comparison to lighter and heavier monogastric herbivores, respectively (Table 3), is linked to the degradation of proteoglycans (such as arabinogalactan-linked proteins) (43, 44). Therefore, it seems that the gut microbiota of (pseudo)ruminants is involved in maximizing energy extraction from food through the 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.

Conclusions. A wide range of studies has suggested that diet and host physiology exert a crucial role in the modulation of both the taxonomical composition and the metabolic repertoire of the mammalian gut microbiota. However, these studies were generally based on 16S rRNA microbial profiling and included a limited number of host species. For this reason, a precise dissection of the peculiar features that characterize gut microbiota functionality in animals with specific dietary habits and an associated digestive system has so far not been performed. In the current study, the gut microbiota composition of 250 fecal samples, corresponding to 77 mammalian species, which broadly cover the mammalian branch of the tree of life, were explored through metagenomic approaches, encompassing 16S rRNA gene microbial profiling and shotgun metagenomics.

Our results demonstrate that diet affects not only intestinal microbial biodiversity but also gut microbiota composition. In detail, 16S rRNA gene microbial profiling underlined the existence of diet-associated genera, suggesting extensive coevolution of gut bacteria with their hosts in order to promote selection of specific taxa. The finding that bacterial taxa typical of mammals following a specific diet cooccur in the gut environment supports this notion. Moreover, prediction of the metabolic potential of the gut microbial population of 24 mammals and metatranscriptome reconstruction of four carnivores and four herbivores revealed that the mammalian gut microbiome evolved to cooperate with its host digestive system from a functional point of view, strengthening the idea that the gut microbiota developed to optimize energy extraction from food. Indeed, among the herbivores, differences in bacterial biodiversity and taxonomical composition were observed when considering the physiology of their digestive systems. These observations were further confirmed by comparison of the herbivore intestinal metabolic repertoires, showing that differences in the physiology of the digestive system correspond to diverse microbial metabolic capabilities. Together, these results suggest that the mammalian gut microbiota has developed in order to

achieve extensive metabolic interplay aimed at maximizing energy and nutrient extraction based on specific dietary habits. However, the difficulties in collecting a sufficient number of fecal samples to fully represent all the categories of diet and the anatomy of the digestive tract reported affected the outcomes of the present study. In this context, the piscivore group is represented only by certain species of dolphins, thus limiting the acquired knowledge on the composition and metabolic repertoire of this group of animals. Furthermore, several samples were obtained from zoo animals, whose microbial community may be affected by human influence and captivity. Therefore, further investigations aiming to retrieve fecal samples from a large cohort of piscivorous mammalian species as well as from mammals living in their natural environment are required to fully understand how the gut microbiota and its metabolic features coevolved with the host. In addition, a follow-up study aimed at collecting fecal samples from different mammals at different time points may be useful to better assess whether the observed differences persist over time or if they are the results of transient shifts.

MATERIALS AND METHODS

Ethics approval and consent to participate. All experimental procedures and protocols involving animals were approved by the Veterinarian Animal Care and Use Committee of Parma University and conducted in accordance with the European Community Council Directives dated 22 September 2010 (2010/63/UE). Human participants gave their informed written consent before enrollment. All investigations were carried out in accordance with the principles of the Declaration of Helsinki.

Sample collection. A total of 250 stool samples were collected through a collaboration with several Italian zoological parks and farms. In the case of aquatic mammals, sample collection was performed during a routine veterinary examination through rectal swabs to avoid contamination (see Table S1 in the supplemental material). Conversely, for all other terrestrial mammals, fecal samples were collected immediately after defecation. To be included in the study, animals had to be healthy, not having undergone treatment with any probiotics or drugs, such as antibiotics, during the six previous months (Table S1). In all cases, an aliquot of each fecal sample was transferred into a fecal container with RNAlater. All samples were kept on ice and shipped to the laboratory under frozen conditions, where they were preserved at −80°C until they were processed.

Bacterial DNA extraction, 16S rRNA gene PCR amplification, and sequencing. Aliquots of fecal samples collected without RNAlater were subjected to bacterial DNA extraction using the QIAamp DNA stool minikit in accordance with the manufacturer's extraction procedure (Qiagen). Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni/Probio_Rev targeting the V3 region of the 16S rRNA gene sequence (45). Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons, which were further processed by using the 16S metagenomic sequencing library preparation protocol (part no. 15044223 Rev. B; Illumina). Amplifications were carried out using a Veriti thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a 2200 TapeStation instrument (Agilent Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic purification step employing Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. The DNA concentration of the amplified sequence library was determined by a fluorometric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM, and 5-μl quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final library. Sequencing was performed using an Illumina MiSeq sequencer with MiSeq reagent kit v3 chemicals.

16S rRNA microbial profiling analysis. The fastq files were processed using a custom script based on the QIIME software suite (46). Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and a mean sequence quality score of $>$20, while sequences with homopolymers of $>$7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures (alpha- and beta-diversity indices, UniFrac analysis), 16S rRNA operational taxonomic units (OTUs) were defined at 100% sequence homology using DADA2 (47); OTUs not encompassing at least two sequences of the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single nucleotide accuracy (46). All reads were classified to the lowest possible taxonomic rank using QIIME2 (46, 48) and a reference data set from the SILVA database v.132 (49). Biodiversity within a given sample (alpha diversity) was calculated considering the observed OTUs for 10 subsamplings of the total read pool. Similarities between samples (beta diversity) were calculated by unweighted/weighted UniFrac and Bray-Curtis (50). The range of similarities is calculated between values 0 and 1. PCoA representations of beta diversity were performed using QIIME2 (46, 48).

Shotgun metagenomics. The extracted DNA was prepared using the Illumina Nextera XT DNA library preparation kit. Briefly, the DNA samples were enzymatically fragmented, barcoded, and purified involving magnetic beads. Then, samples were quantified using the fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 TapeStation instrument (Agilent Technologies, USA),

and normalized to 4 nM. Sequencing was performed using an Illumina NextSeq 500 sequencer with NextSeq high-output v2 kit chemicals (150 cycles).

Analysis of metagenomic data sets. The obtained fastq files were filtered for reads with a quality score of <25, for reads of >80 bp, and for sequences of the mammalian host DNA. 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 further analysis with METAnnotatorX using default settings (51). Investigation of glycosyl hydrolase (GH) profiles together with the reconstruction of bacterial metabolic pathways and evaluation of their abundance in the shotgun metagenomics data sets were assessed using custom scripts based on RapSearch2 software (52) and the CAZy database or the MetaCyc database (53), respectively.

RNA extraction. RNAlater-preserved stool samples were vortexed and homogenized after thawing for 10 min. Approximately 0.4 g of stool slurry was mixed with 1 ml of QIAzol lysis reagent (Qiagen, UK) in a sterile tube containing glass beads (Merck, Germany). The cells were lysed by alternating 2 min of stirring the mix on a Precellys 24 homogenizer (Bertin instruments, France) with 2 min of static cooling; this step was repeated three times. The lysed cells were centrifuged at 12,000 rpm for 15 min, and the upper phase was recovered. The RNA samples were purified using the RNeasy minikit (Qiagen, UK) by following the manufacturer's protocol. RNA concentration and purity were evaluated using a Picodrop microliter spectrophotometer (Picodrop, UK).

RNA sequencing analysis performed by NextSeq Illumina. For RNA sequencing, 2.5 μg of total RNA was treated to remove rRNA by using the Ribo-Zero magnetic kit (Illumina), followed by purification of the rRNA-depleted sample by ethanol precipitation. RNA was processed according to the manufacturer's protocol. The yield of rRNA depletion was checked by use of a 2200 TapeStation (Agilent Technologies, USA). Then, a whole transcriptome library was constructed using the TruSeq stranded RNA LT kit (Illumina). Samples were loaded into a NextSeq high-output v2 kit (150 cycles) (Illumina) as indicated by the technical support guide. The reads were depleted of adapters and quality filtered (with overall quality, quality window, and length filters). Sequences corresponding to hosts' genomes were removed through mapping with bwa software (54) against a custom database of hosts' genomes. Retained reads were submitted to analysis with the METAnnotatorX tool (51). Investigation of GH profiles together with the reconstruction of bacterial metabolic pathways and evaluation of their abundance in the shotgun metagenomics data sets were assessed using custom scripts based on RapSearch2 software (52) and the CAZy database or the MetaCyc database (53), respectively.

Statistical analysis. All statistical analyses, i.e., analysis of variance (ANOVA), permutational multivariate analysis of variance (PERMANOVA), Student's *t* test, and the Kendall tau rank covariance analysis were performed with SPSS software v. 22 (SPSS Statistics for Windows, version 22.0; IBM Corp., Armonk, NY). The force-driven network was created using Gephi (https://gephi.org/), and modularity was defined with a resolution of 0.6.

Data availability. Raw sequences of 16S rRNA gene profiling data coupled with shotgun metagenomics and RNA sequencing data are accessible through SRA study accession numbers PRJNA545289 and PRJNA545214.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.4 MB.

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We declare that we have no competing interests.

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