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

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1 **Deciphering the impact of diet and host physiology on the mammalian gut microbiome by**
2 **multi-omics approaches**

4 Running title: the mammalian gut microbiota and diet

5 Key words: microbiota, metagenomics, mammals, metatranscriptomics, diet, physiology

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26 **Abstract**

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

41 **Importance**

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

- 51 both metabolic features and transcriptional responses of the intestinal bacterial community based on
- 52 different diets.

53 Introduction

54 The functional roles exerted by the mammalian gut microbiota have in recent years been scrutinized
55 by a range of studies focusing on multiple aspects of host biology, including the immune, digestive
56 and nervous systems (1-4). In this regard, gut microbiota composition has been shown to be
57 influenced by host genetics (5-11) as well as environmental factors that are linked to host lifestyle
58 and diet (7, 10, 12-14). Microbe-host interactions are the result of intricate adaptive occurrences,
59 through a process known as host-microbe co-evolution, being responsible for the adaptation of
60 mammals to new environmental niches and having contributed to their dispersal and current global
61 distribution (5, 15). Among multiple factors, diet, host evolutionary history and host physiology are
62 currently presumed to be the main drivers implicated in the modulation of the mammalian gut
63 microbiota (5, 7, 12, 13, 16-18). In this context, several comparative analyses of mammalian gut
64 microbial communities have revealed associations between the composition of the gut ecosystem
65 and host diet, even among phylogenetically un-related hosts (5, 18), and supported the notion that
66 diet contributes to the microbiome plasticity by selecting particular metabolic activities to allow
67 degradation of specific components of the host diet (5, 18, 19). Specifically, while carnivorous
68 communities were reported to be specialized in the degradation of proteins, herbivorous
69 microbiomes harbour genes which encode enzymatic activities involved in the breakdown of
70 complex plant-derived polysaccharides, and absent in the genetic repertoire of their host, and which
71 synthesize amino acid building blocks to cope with protein deficiency typical of their diet (18, 20,
72 21). In concert with diet, host phylogeny and physiology have been proposed as other crucial
73 factors affecting the mammalian gut microbial community (5, 10, 16, 17). In recent years, the term
74 'phylosymbiosis' has been proposed to define the eco-evolutionary pattern that associates host
75 evolutionary changes with ecological modulations of their intestinal microbial community (22, 23).
76 Indeed, despite the inter-individual fluctuations of gut microbiomes and the possible rapid changes
77 in response to diet and environment, it has been demonstrated that the mammalian gut microbiota

78 composition diverges at a relatively constant rate across an evolutionary timescale (10, 24),
79 suggesting that host traits that undergo changes across host phylogeny, including gut physiology,
80 have an important role in shaping the intestinal microbial community across mammals (7).
81 However, this conserved pattern of host-microbe phylosymbiosis seems to be restricted to
82 mammals. Indeed, meta-analysis performed on fecal samples of various bird, fish, reptile or
83 amphibian species failed to report the same strict correlation (7, 16). Altogether, these findings
84 indicate that the gut microbiota plays a pivotal role in facilitating adaptation to dietary changes
85 adopted by mammals as part of their evolution, revealing particular correlations between a given
86 gut microbiota and their associated host diet and/or digestive system (5, 7, 9, 10). Nevertheless,
87 despite many studies depicting the gut microbiota as a hidden organ that exerts key metabolic
88 activities to support its host, the composition and especially the functional role of mammalian gut
89 microbial populations has not been fully explored. Indeed, despite the extensive number of
90 mammalian species involved, most of the available studies explored the mammalian gut microbiota
91 composition exclusively through 16S rRNA microbial profiling, thus failing to provide a correlation
92 between the composition of the mammalian gut microbiota and its (predicted) metabolic functions
93 (5, 7, 10, 16, 25). Other studies, even though they were based on shotgun metagenomics, did not
94 investigate transcriptional profiles of the collected samples. In this context, in order to expand our
95 knowledge in this field, the specific taxonomic and functional traits associated with different diets
96 and physiology of the host's digestive system across the mammalian branch of the tree of life were
97 assessed by means of metagenomics (16S rRNA microbial profiling and shotgun metagenomics)
98 and metatranscriptomics approaches. Specifically, we collected fecal samples from 250 mammals,
99 covering 77 species and representing a broad range of mammalian biodiversity. These samples were
100 subjected to 16S rRNA gene microbial profiling in order to obtain an overview of the taxonomic
101 composition of the gut microbiota among their mammalian hosts. Moreover, 24 key samples were
102 subjected to shotgun metagenomic sequencing and reconstruction of their microbial metabolic

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

107 Results and discussion

108 **The gut microbiota biodiversity across the mammalian branch of the tree of life.** We
109 performed 16S rRNA gene-based microbial profiling of 250 fecal samples corresponding to 77
110 mammalian species, together forming a broad coverage of the mammalian tree of life (Table S1)
111 (Supplementary Excel File 1). Specifically, the enrolled mammalian species represent 66
112 omnivores, 63 carnivores, 115 herbivores (encompassing different sub-classes accordingly to the
113 physiology of their digestive tract) and 6 piscivores (Table 1). In this context, because of the
114 difficulties in collecting multiple fecal samples from non-domesticated mammals, some of the fecal
115 samples were collected from wild animals (i.e. wolves or boars) while others were retrieved from
116 animals raised in captivity. Furthermore, difficulties in collecting fecal samples from aquatic
117 mammals significantly restricted the number of piscivore members, being limited to two species of
118 dolphins (three fecal samples per dolphin species) (Table S1). Illumina sequencing produced a total
119 of 15,307,128 reads, with an average of 61,229 reads per sample. Evaluation of the alpha-diversity,
120 i.e. the biodiversity of the bacterial population harboured by each sample, was performed through
121 rarefaction curves representing the number of observed OTUs generated with 100 % identity cut-off
122 and obtained for 10 sub-samplings of the total read pool. Average curves obtained for the 28
123 mammalian taxonomic families included in this study revealed that some herbivorous mammalian
124 species, i.e. *Equidae*, *Camelidae*, *Macropodidae*, *Bovidae*, *Elephantidae* and *Giraffidae* possess a
125 higher gut bacterial biodiversity compared to that of other mammals, supported by Student's t-test
126 p -value of <0.001 (Figure 1a). This observation is confirmed by average diet-based rarefaction
127 curves revealing a significantly higher biodiversity (Student's t-test p -value of <0.001) of the gut
128 microbiota of herbivores when compared to that of omnivores or carnivores (the latter including
129 piscivores) (Figure S1a). These data indicate that the overall bacterial biodiversity harboured by the
130 mammalian gut positively correlates with the abundance of plant-based foods in the diet (p -value $<$
131 0.001), suggestive of a major metabolic role played by bacteria in the gastrointestinal tract of

herbivores. In this context, we also performed a sub-classification of the enrolled herbivores based on the physiology of their digestive system (Table S1) (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 multi-chambered stomach (26). The only exception was represented by *hippopotamidae* that showed lower biodiversity. Notably, this apparent inconsistency may reflect the peculiar physiology of the three-chambered stomach of these non-ruminant herbivores (26). 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 when compared to 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 (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 the encumbrance of a large hindgut, thus being advantageous for small animals with high ratio of food intake with respect to their size (26). In contrast, in colon fermenters the content of colon and cecum mix freely and act as a single fermentation site (26), 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 genus level were used to perform a beta-diversity analysis using the Bray-Curtis distance matrix, and then represented by means of a PCoA plot (Figure 1b) (Figure

157 S2a). This analysis revealed clustering of samples based on taxonomic family, as expected, with
158 overlap of families with a similar diet (Figure S2a). In fact, re-colouring of the samples based on
159 dietary habits revealed that herbivores, omnivores and carnivores (including piscivores) clustered
160 separately (Figure 1b), with herbivores forming sub-clusters, confirming previously published
161 observations (18). In order to detail differences between herbivores, a specific Bray-Curtis PCoA
162 was generated (Figure S2b). The latter revealed three major clusters constituted by i) polygastric
163 ruminants and pseudo-ruminants (Tylopoda), ii) heavier monogastric herbivores, and iii) lighter
164 monogastric herbivores and *hippopotamidae* (Figure S2b). These findings highlight that diet, as
165 well as the physiology and anatomy of the herbivorous digestive system, not only impact on the
166 overall bacterial biodiversity, i.e., number of different bacterial taxa, but also on the gastrointestinal
167 microbiota composition.

168 Furthermore, in depth analysis of the microbial taxonomic profiles reconstructed from 16S rRNA
169 gene-based microbial profiling data evidenced similarities between taxonomic families of mammals
170 with an analogous diet (Figure 2a). Details regarding key taxa correlated with specific diets or
171 gastrointestinal physiologies are extensively discussed in the supplementary text. Amongst the most
172 relevant findings, it is worth mentioning that carnivores and herbivores are characterized by a
173 peculiarly high average abundance of the genus *Fusobacterium* and members of the
174 *Ruminococcaceae* family, respectively (Figure 2b). In this context, it has previously been shown
175 that the *Fusobacterium* genus is generally associated with a protein-rich diet (27), while a high
176 abundance of members of the *Ruminococcaceae* family is related to a fibre-based diet, since the
177 latter are degraders of a wide range of carbohydrates (28). Nevertheless, though our findings
178 indicate that members of these two bacterial taxonomic groups play a defining metabolic role for
179 their host, their sub-genus phylogeny and genetic potential are still poorly characterized. They
180 therefore represent prime targets for further genomic and functional studies. In this regard, analysis
181 of the herbivorous gut microbiota revealed that the *in silico* predicted genera UCG-005 and UCG-

010 of family *Ruminococcaceae* together represent 18.49 % of the total gut microbial population of polygastric herbivores (Figure S3a-b). Moreover, the small monogastric class (<100 Kg-average body weight) is characterized by a higher number of class-specific taxa when compared to 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 (26).

189

Co-variance 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 co-operative and competitive behaviours 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 co-variance analysis using all taxonomic profiles obtained in this study. Data collected were used to construct a force-driven network where attractive and repulsive forces between nodes correspond to positive and negative co-variances 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 co-occurring 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) (Figure 3b) revealed that genera more abundant in herbivores, carnivores and piscivores clustered together, thus suggesting the existence of putative co-operational 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

207 omnivorous mammals. This finding may indicate that omnivores are not associated with specific
208 bacterial genera (or *vice versa*), but, rather, possess a combination of bacterial taxa typical of
209 herbivores and carnivores. This notion is in accordance with a previous observation that omnivores
210 do not possess 'generalist' bacterial lineages able to digest both plant- and animal-derived
211 compounds but rather a combination of herbivorous and carnivorous specialist bacterial groups
212 (25). In this milieu, it seems that diet may play a major role in modulating the mammalian gut
213 microbiota, resulting in efficient metabolism of dietary food components.

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

225

226 **Functional characterization of the mammalian gut microbiota.** The 16S rRNA gene-based
227 microbial profiling analysis revealed substantial differences in the taxonomic composition of the
228 250 collected fecal samples based on diet and physiology of the digestive system. For this reason, in
229 order to trace potential differences in the functional repertoire of mammalian gut microbial
230 populations, a shotgun metagenomic approach was performed for 24 fecal samples. Specifically, to
231 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 Supplementary Text). Furthermore, statistical analysis revealed that carnivores possess a lower number of pathways with differential (higher or lower) abundance when compared to 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 when compared 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 super-pathway of trimethylamine degradation (Table S5). Notably, choline, a quaternary amine principally found in meats, is known as 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 which 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 community, with a consequent impact of the metabolic pathways encoded by these mammalian intestinal microbial communities.

Differences between the gut glycobiome 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 Supplementary Text. Focusing on the comparison between the glycobiome profiles of carnivores and herbivores, we performed a Student's t-test statistical analysis. Results revealed that a large number of GH families possess differential abundance between the representatives of the two considered diets (Table S6). In this context, a marked commitment of carnivores was noticed towards the degradation of animal-derived host glycans and their degradation products (GH20, GH33, GH92, GH101, GH123, GH125 and GH129) 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 highlighted also commitment toward degradation of fungal polysaccharides such as mycodextran (GH87) (Table S6).

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

287

288 **Metatranscriptomic analysis of Carnivores and Herbivores microbiomes.** Metagenomics data
289 provided interesting information regarding functional commitment of the gut microbiota of
290 herbivores and carnivores towards metabolism of specific dietary components. In order to evaluate
291 if transcriptional profiles of these microbiomes reflect such observations, we performed
292 metatranscriptome analysis of fecal samples from four carnivores and four herbivores (Table S7),
293 which were selected in order to represent various animal genera. Sequenced metatranscriptome
294 datasets were processed for removal of host DNA through mapping against a custom database of
295 host genomes resulting in a total of 38,921,420 reads with an average of 4,865,177 reads per sample
296 and the latter were further subjected to prediction of the expressed glycobiome and repertoire of
297 degradation pathways (Table S5, Table S6 and Figure 5).

298 Inspection of transcriptional data revealed that the range of pathways involved in the breakdown of
299 typical plant carbohydrates, i.e. xylose, arabinose and starch, found to be more abundant in
300 herbivores based on shotgun metagenomic data (Table S5), are also more expressed in animals
301 following this diet (Table S5). Similarly, analysis of the expressed glycobiomes focusing on GH
302 families showing differential abundance in metagenomic data, evidenced that genetic members of
303 the GH9, GH26, GH39, GH43, GH51, GH67 and GH74 glycosyl hydrolase families, predicted to
304 be involved in the breakdown of plant-related carbohydrates, are more expressed in herbivores. In
305 contrast, genes encoding GH20, GH33 and GH129 family enzymes, which are predicted to be
306 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 (Figure 5a). These analyses of transcriptomics data revealed that, compared 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, 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 datasets 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 (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 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

332 to cover the four main classes of herbivores depicted by analysis of 16S rRNA gene microbial
333 profiling data, i.e. polygastric ruminants, polygastric pseudo-ruminants (Tylopoda), heavier
334 monogastric herbivores (>100 Kg of average body weight) and lighter monogastric herbivores
335 (<100 Kg of average body weight). Notably, comparison of the gut microbiome of polygastric
336 ruminants and pseudo-ruminants revealed very limited differences in terms of encoded pathways
337 and predicted glycobiome (Table S8 and Table S9). In detail, only one metabolic pathway with
338 relative abundance >0.001 % was found to show increased abundance in ruminants ± 50 % when
339 compared to pseudo-ruminants (Student's t-test p-value <0.05), i.e. L-glutamate degradation IX
340 (+72.89 %) (Table S8). Moreover, no degradation pathway classes showed statistically significant
341 differential abundance. Notably, these data are consistent with the previously proposed notion that
342 the gut microbiota of these two families of herbivores with a similar multi-chambered digestive
343 system may exert comparable metabolic functions (26, 38). Indeed, comparison of the number of
344 pathways with a statistically significant different abundance between the two groups of monogastric
345 herbivores and ruminants or pseudo-ruminants revealed similar trends with the only exception of a
346 slight decrease in the number of pathways with statistically significant higher abundance in the
347 pseudo-ruminants when compared to monogastric herbivores (Table S10). For this reason,
348 ruminants and pseudo-ruminants were considered as a single group for further comparison with
349 heavier monogastric and lighter monogastric herbivores. Metabolic pathway prediction revealed
350 that the total number of pathways with an abundance of >0.001 % and the number of degradative
351 pathways with an abundance of >0.001 % is lower in polygastric animals when compared to
352 monogastric herbivores.

353 Furthermore, our collected data revealed that the gut microbiota of ruminants and pseudo-ruminants
354 encode the highest number of pathways with significant lower abundance when compared to
355 monogastric herbivores (Figure 6), with a similar trend observed for degradative pathways (Figure
356 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 when compared to 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 (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 (26). 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 when compared 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 (ANOVA post-hoc Bonferroni p-value <0.05 when compared to either of the other groups) was performed (Table S10). Notably, the gut microbiota of the heavier monogastric herbivores revealed a specific commitment towards 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 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

382 efficient utilization of core plant saccharides. Furthermore, when considering polygastric
383 herbivores, in addition to a higher abundance of pathways for degradation of simple sugars (mono-
384 or di-saccharides) such as D- and L-arabinose, fucose, maltose, melibiose, trehalose and xylose, this
385 herbivore class showed a higher abundance of a wide range of amino acid degradation pathways
386 (Table S10). Notably, these results suggest that the mammalian gut microbiota plays a significant
387 role in performing specific metabolic tasks not only dependent on host diet but also on the
388 physiology of the corresponding digestive system.

389 Further exploration of the metabolic potential of herbivores through analysis of their glycobiome
390 revealed that the microbiome of lighter monogastric herbivores encode the highest number of GH
391 families at a significantly higher abundance (Table 3). Furthermore, five of the six GH families
392 enriched in fecal material of lighter monogastric herbivores are either predicted to represent
393 chitinase activity (associated with GH19), which participate in the hydrolysis of (1→4)- β -linkages
394 between N-acetyl-D-glucosamine residues in the chitin-derived chitodextrins (GH25 and GH73),
395 induce breakdown of 1,3- β -glucans (GH81) or encode broad spectrum β -glucosidases and β -
396 mannosidases (GH1). In this context, all these predicted enzymatic activities may suggest a genetic
397 specialization toward degradation of the main fungal cell wall components (42) (Table 3).
398 Moreover, three of the four GH families enriched in heavier monogastric herbivores are involved in
399 xylan degradation (GH54, GH116 and GH120) (Table 3). Therefore, these data may indicate that the
400 gut microbiota of heavier monogastric herbivores has adapted to compensate for the reduced
401 capability of these animals to metabolize complex plant saccharides when compared to polygastric
402 ruminants. Furthermore, the abundance of GH family 79, which is enriched in polygastric
403 herbivores (by 803 % and 3981 %) when compared to lighter and heavier monogastric herbivores,
404 respectively (Table 3), is linked to the degradation of proteoglycans (such as arabinogalactan-linked
405 proteins) (43, 44). Therefore, it seems that the gut microbiota of (pseudo)ruminants is involved in

406 maximizing energy extraction from food through improved breakdown of the extracellular matrix of
407 plants.

408 Altogether these data reveal the relevant role of physiology and anatomy of the mammalian
409 digestive system in order to co-operatively achieve optimal energy extraction from their particular
410 diet.

411

412 **Conclusions**

413 A wide range of studies has suggested that diet and host physiology exert a crucial role in the
414 modulation of both the taxonomical composition and metabolic repertoire of the mammalian gut
415 microbiota. However, these studies did focus on specific diets and included a limited number of
416 host species. For this reason, a precise dissection of the peculiar features that characterize the gut
417 microbiota functionality in animals with specific dietary habits and an associated digestive system
418 has so far not been performed. In the current study, the gut microbiota composition of 250 fecal
419 samples, corresponding to 77 mammalian species, which broadly cover the mammalian branch of
420 the tree of life, were explored through metagenomic approaches, encompassing 16S rRNA gene
421 microbial profiling and shotgun metagenomics. Our results demonstrate that diet not only affects
422 the intestinal microbial biodiversity but also the gut microbiota composition. In detail, 16S rRNA
423 gene microbial profiling underlined existence of diet-associated genera, suggesting extensive co-
424 evolution of gut bacteria with their hosts in order to promote selection of specific taxa. The finding
425 that bacterial taxa typical of mammals following a specific diet co-occur in the gut environment
426 supports this notion. Moreover, prediction of the metabolic potential of the gut microbial population
427 of 24 mammals and metatranscriptome reconstruction of four carnivores and four herbivores
428 revealed that the mammalian gut microbiome evolved to co-operate with its host digestive system
429 from a functional point of view, strengthening the idea that the gut microbiota developed to
430 optimize energy extraction from food. Indeed, among the herbivores, differences in the bacterial

biodiversity and taxonomical composition were observed when considering the physiology of their digestive system. These observations were further confirmed by comparison of the herbivore intestinal metabolic repertoire, showing that differences in the physiology of the digestive system correspond to diverse microbial metabolic capabilities. Altogether, these results suggest that 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 co-evolved 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 following the principles of the Declaration of Helsinki.

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

464 **Bacterial DNA extraction, 16S rRNA gene PCR amplification and sequencing.** Aliquots of
465 fecal samples collected without RNAlater were subjected to bacterial DNA extraction using the
466 QIAamp DNA Stool Mini Kit following the manufacturer's extraction (Qiagen). Partial 16S rRNA
467 gene sequences were amplified from extracted DNA using primer pair Probio_Uni/Probio_Rev.
468 targeting the V3 region of the 16S rRNA gene sequence (45). Illumina adapter overhang nucleotide
469 sequences were added to the partial 16S rRNA gene-specific amplicons, which were further
470 processed involving the 16S Metagenomic Sequencing Library Preparation Protocol (Part
471 #15044223 Rev. B – Illumina). Amplifications were carried out using a Verity Thermocycler
472 (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a
473 2200 TapeStation Instrument (Agilent Technologies, USA). DNA products obtained following
474 PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic
475 purification step employing the Agencourt AMPure XP DNA purification beads (Beckman Coulter
476 Genomics GmbH, Bernried, Germany) in order to remove primer dimers. DNA concentration of the
477 amplified sequence library was determined by a fluorometric Qubit quantification system (Life
478 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 reads 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 mean sequence quality score >20 while sequences with homopolymers >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 2 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 dataset from the SILVA database v.132 (49). Biodiversity within a given sample (alpha-diversity) was calculated considering the observed OTUs for 10 sub-samplings 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 following 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 fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 Tape Station Instrument (Agilent Technologies, USA) and normalized to 4nM. Sequencing was performed using an Illumina NextSeq 500 sequencer with NextSeq High Output v2 Kit Chemicals 150 cycles.

Analysis of metagenomic datasets. The obtained fastq files were filtered for reads with a quality of < 25, for reads > 80 bp and for sequences of the mammalian host DNA. Moreover, bases were

504 removed from the end of the reads unless the average quality score was > 25, in a window of 5 bp.
505 Only paired data were used to further analysis with METAnnotatorX using default settings (51).
506 Investigation of Glycosyl Hydrolase (GH) profiles together with the reconstruction of bacterial
507 metabolic pathways and evaluation of their abundance in the shotgun metagenomics datasets were
508 assessed using custom scripts based on RapSearch2 software (52) and the CAZy database or the
509 MetaCyc database (53), respectively.

510 **RNA extraction.** RNAlater-preserved stool samples were vortexed and homogenized after thawing
511 for 10 min. Approximately 0.4 g of stool slurry was mixed with 1 mL of QIAzol Lysis Reagent
512 (Qiagen, UK) in a sterile tube containing glass beads (Merck, Germany). The cells were lysed
513 alternating 2 minutes of stirring the mix on a Precellys 24 homogenizer (Bertin instruments, France)
514 with 2 minutes of static cooling; this step was repeated three times. The lysed cells were centrifuged
515 at 12,000 rpm for 15 min and the upper phase was recovered. The RNA samples were purified
516 using the RNAeasy Mini Kit (Qiagen, UK) following the manufacturer's protocol. RNA
517 concentration and purity were evaluated by a Picodrop microliter spectrophotometer (Picodrop,
518 UK).

519 **RNAseq analysis performed by NextSeq Illumina.** For RNA sequencing, 2.5 µg of total RNA
520 was treated to remove ribosomal RNA by the Ribo-Zero Magnetic Kit (Illumina), followed by
521 purification of the rRNA-depleted sample by ethanol precipitation. RNA was processed according
522 to the manufacturer's protocol. The yield of rRNA depletion was checked by a Tape station 2200
523 (Agilent Technologies, USA). Then, a whole transcriptome library was constructed using the
524 TruSeq Stranded RNA LT Kit (Illumina). Samples were loaded into a NextSeq High Output v2 Kit
525 Chemicals 150 cycles (Illumina) as indicated by the technical support guide. The reads were
526 depleted of adapters, quality filtered (with overall quality, quality window and length filters).
527 Sequences corresponding to hosts' genomes were removed through mapping with bwa software
528 (54) against a custom database of hosts' genomes. Retained reads were submitted to analysis with

529 METAnnotatorX tool (51). Investigation of Glycosyl Hydrolase (GH) profiles together with the
530 reconstruction of bacterial metabolic pathways and evaluation of their abundance in the shotgun
531 metagenomics datasets were assessed using custom scripts based on RapSearch2 software (Zhao et
532 al 2012) and the CAZy database or the MetaCyc database (Caspi et al 2012), respectively.

533 **Statistical analysis.** All statistical analyses, i.e. ANOVA, PERMANOVA, Student's t-test as well
534 as the Kendall tau rank co-variance analysis were performed with SPSS software v. 22 (IBM SPSS
535 Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The force-driven network was
536 created using Gephi (<https://gephi.org/>) and modularity was defined with resolution of 0.6.

537 **Availability of data and materials.** Raw sequences of 16S rRNA gene profiling data coupled with
538 shotgun metagenomics and RNA sequencing data are accessible through SRA study accession
539 number PRJNA545289 (<https://www.ncbi.nlm.nih.gov/bioproject/545289>) and PRJNA545214
540 (<https://www.ncbi.nlm.nih.gov/bioproject/545214>).

541

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560 **Competing interests**

561 The authors declare that they have no competing interest.

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703

704

705 **Tables**

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

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

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

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719 **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%

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

722 **Table 3:** List of GH families with statistically significant higher or lower abundance based on
 723 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%

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

726 **Figure legends**

727

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

733

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

740

741 **Figure 3.** Co-variance force-driven network of genera profiled with relative abundance of >5 % in
742 at least a sample. Nodes represent genera included in the analysis and attraction and repulsion
743 forces are proportional to statistically significant co-variances and co-exclusions obtained using the
744 Kendall’s tau correlation coefficient. Node size is proportional to the number of correlations. Panel
745 a reports the network with nodes colored based on the predicted modularity class (using 0.6
746 resolution). Panels b and c show the same network with nodes colored to highlight bacterial genera
747 identified as more abundant in a specific diet through analysis of carnivores, herbivores, piscivores
748 and omnivores as well as between only carnivores and herbivores, respectively. Figure numerical
749 legend: *Acinetobacter* (1), *Actinobacillus* (2), *Aeromonas* (3), *Akkermansia* (4), *Alistipes* (5),
750 *Allobaculum* (6), *Alloprevotella* (7), *Anaerococcus* (8), *Asteroleplasma* (9), *Bacillus* (10),
751 *Bacteroides* (11), *Barnesiella* (12) *Bifidobacterium* (13), *Blautia* (14), *Brevundimonas* (15), CAG-

752 352 (16), *Carnobacterium* (17), *Catenibacterium* (18), *Catenisphaera* (19), *Cellulosilyticum* (20),
753 *Cetobacterium* (21), *Christensenellaceae* R-7 group (22), *Clostridium sensu stricto* 1 (23),
754 *Collinsella* (24), *Comamonas* (25), *Corynebacterium* 1 (26), *Cutibacterium* (27), *Dialister* (28),
755 *Enterococcus* (29), *Epulopiscium* (30), *Erysipelotrichaceae* UCG-002 (31), *Erysipelotrichaceae*
756 UCG-004 (32), *Escherichia-Shigella* (33), *Eubacterium coprostanligenes* group (Ruminococcaceae
757 family) (34), *Faecalibacterium* (35), *Faecalibaculum* (36), Family XIII AD3011 group
758 (Clostridiales order) (37), *Fibrobacter* (38), *Flavobacterium* (39), *Fusobacterium* (40),
759 *Helicobacter* (41), *Ignatzschineria* (42), *Lachnospira* (43), *Lactobacillus* (44), *Lysinibacillus* (45),
760 *Megamonas* (46), *Megasphaera* (47), *Myoides* (48), *Paenibacillus* (49), *Pedobacter* (50),
761 *Peptoniphilus* (51), *Peptostreptococcus* (52), *Photobacterium* (53), *Prevotella* 2 (54), *Prevotella* 7
762 (55), *Prevotella* 9 (56), *Prevotellaceae* UCG-001 (57), *Prevotellaceae* UCG-003 (58), *Pseudomonas*
763 (59), *Psychrobacter* (60), *Rikenellaceae* RC9 group (61), *Ruminiclostridium* 6 (62),
764 *Ruminococcaceae* NK4A214 group (63), *Ruminococcaceae* UCG-002 (64), *Ruminococcaceae*
765 UCG-005 (65), *Ruminococcaceae* UCG-010 (66), *Ruminococcaceae* UCG-013 (67),
766 *Ruminococcaceae* UCG-014 (68), *Ruminococcaceae* V9D2013 group (69), *Ruminococcus* 1 (70),
767 *Saccharofermentans* (71), *Sarcina* (72), *Shuttleworthia* (73), *Solibacillus* (74), *Solobacterium* (75),
768 *Sphaerochaeta* (76), *Staphylococcus* (77), *Streptococcus* (78), *Streptomyces* (79), *Subdoligranulum*
769 (80), *Succinivibrio* (81) *Sutterella* (82), *Treponema* 2 (83), *Turicibacter* (84), U. m. of Rickettsiales
770 order (85), U. m. of WPS-2 phylum (86), U. m. of Bacteroidales BS11 gut group family (87), U. m.
771 of Bacteroidales order (88), U. m. of Bacteroidales RF16 group family (89), U. m. of Bacteroidales
772 UCG-001 family (90), U. m. of Bacteroidia class (91), U. m. of Burkholderiaceae family (92), U.
773 m. of Caulobacteriaceae family (93), U. m. of Clostridiaceae 1 family (94), U. m. of Clostridiales
774 vadinBB60 group family (96), U. m. of Coriobacteriales order (97), U. m. of Cyanobacteria phylum
775 (98), U. m. of Enterobacteriaceae family (99), U. m. of Erysipelotrichaceae family (100), U. m. of
776 Eukaryota kingdom (101), U. m. of F082 family (102), U. m. of Firmicutes phylum (103), U. m. of

777 Flavobacteriaceae family (104), U. m. of Gammaproteobacteria class (105), U. m. of
778 Lachnospiraceae family (106), U. m. of Lactobacillales order (107), U. m. of Moraxellaceae family
779 (108), U. m. of Muribaculaceae family (109), U. m. of p-251-o5 family (110), U. m. of p-2534-
780 18B5 gut group family (111), U. m. of Pasteurellaceae family (112), U. m. of Peptostreptoocaceae
781 family (113), U. m. of Planococcaceae family (114), U. m. of Prevotellaceae family (115), U. m. of
782 Rhodospirillales order (116), U. m. of Ruminococcaceae family (117), U. m. of
783 Sphingomonadaceae family (118), U. m. of Verrucomicrobiae class (119), U. m. of Weekellaceae
784 family (120), *Vibrio* (121), *Vitreoscilla* (122) and *Yersinia* (123).

785

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

793

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

801

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

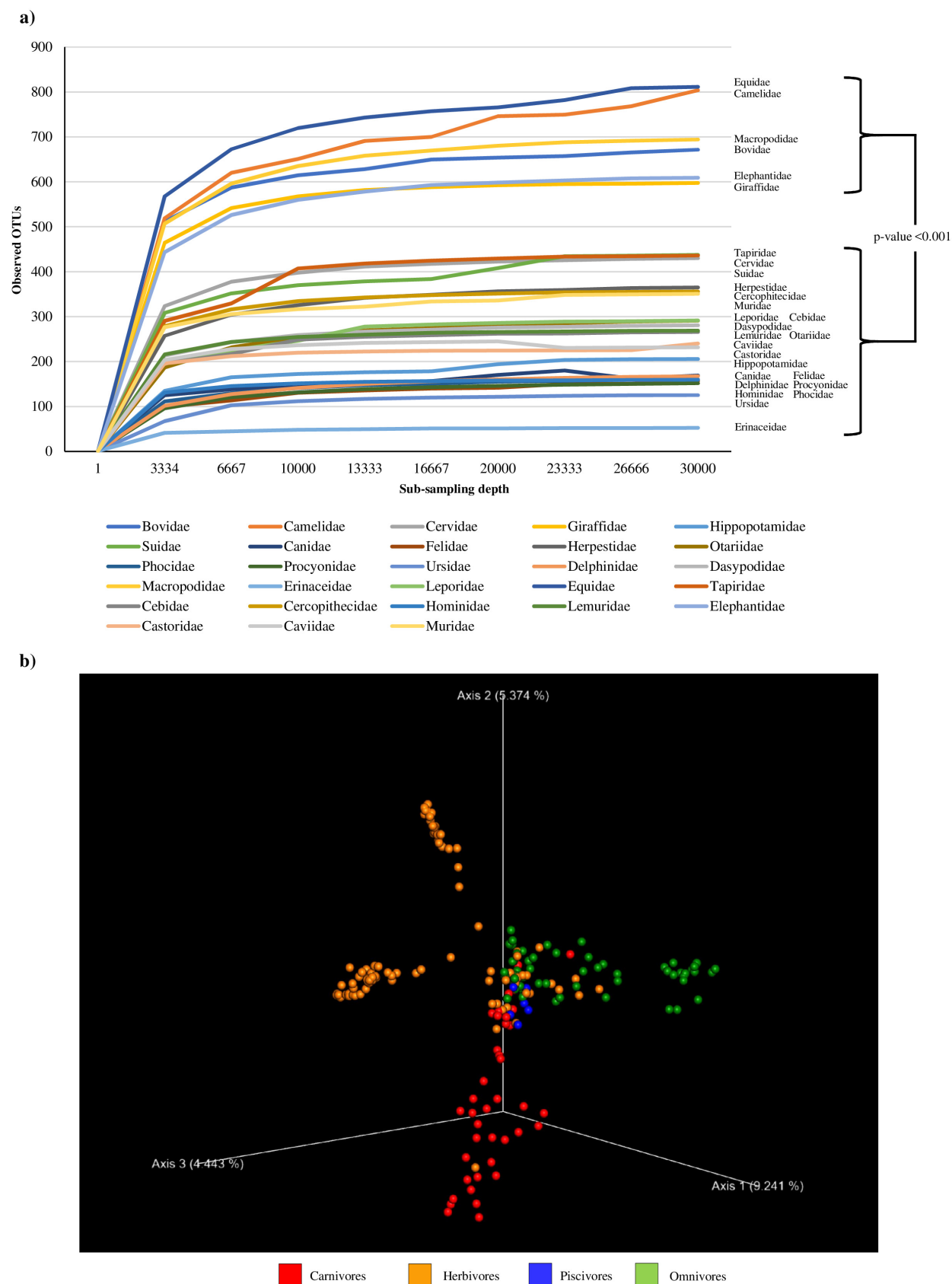


Figure 1

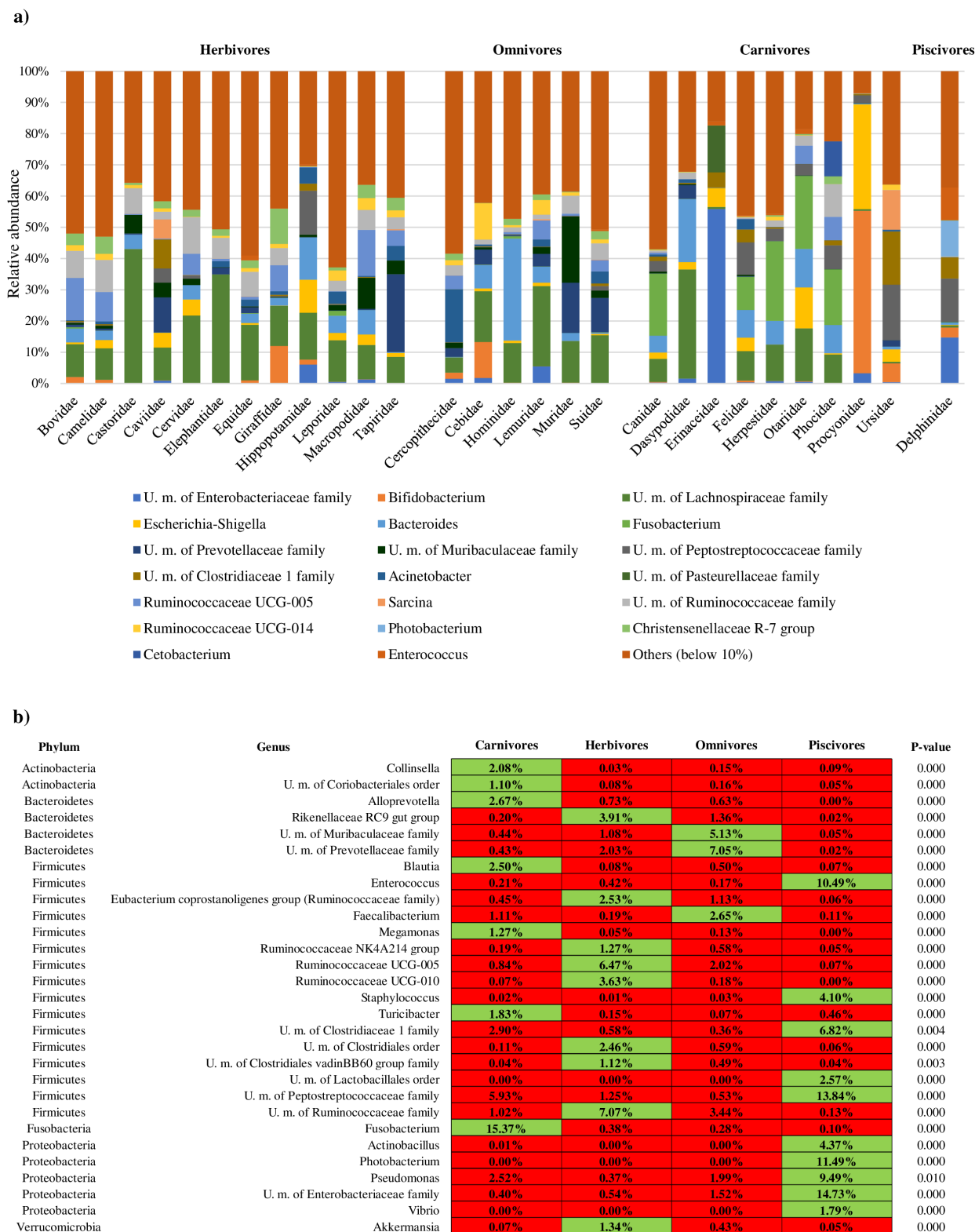
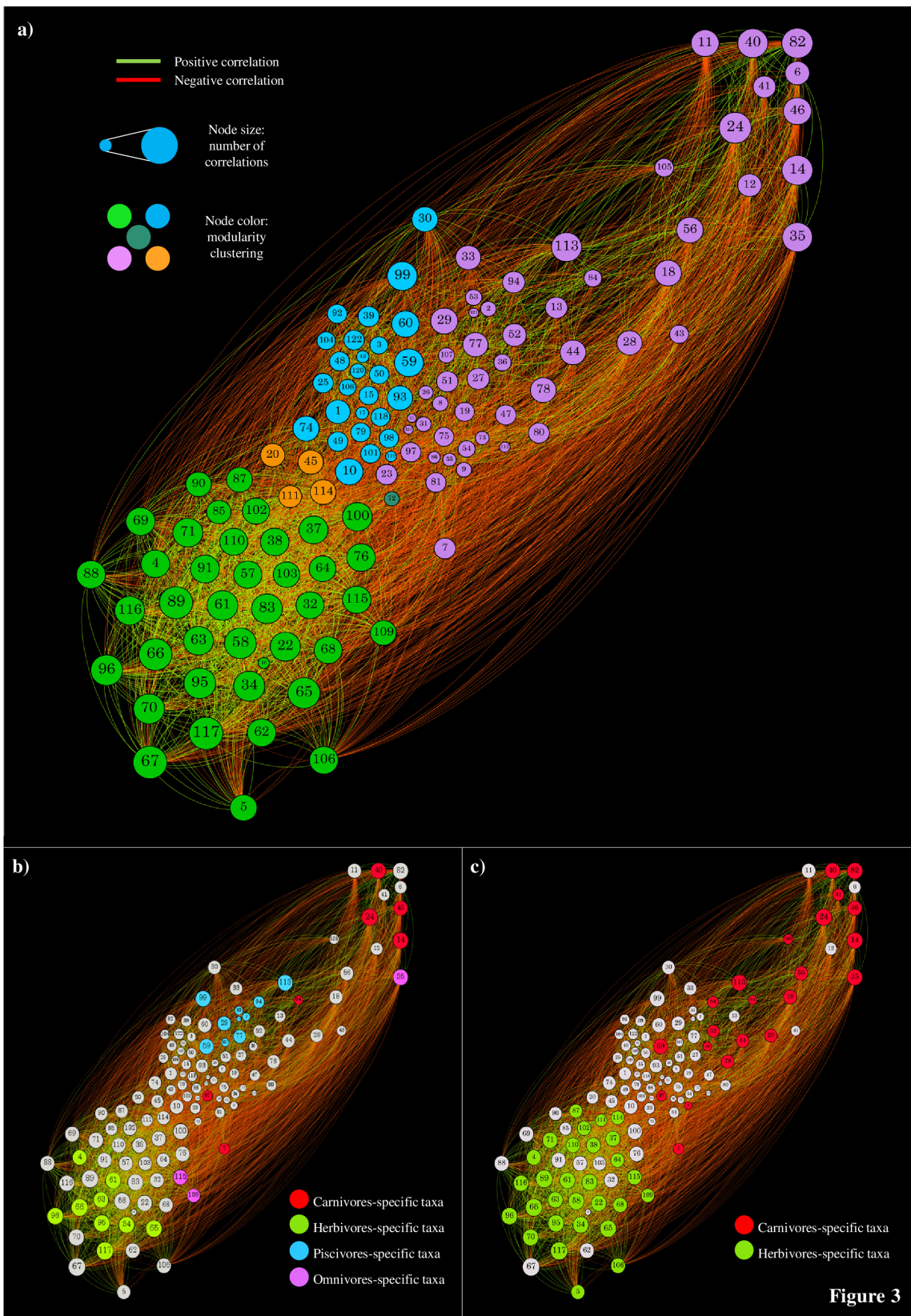


Figure 2



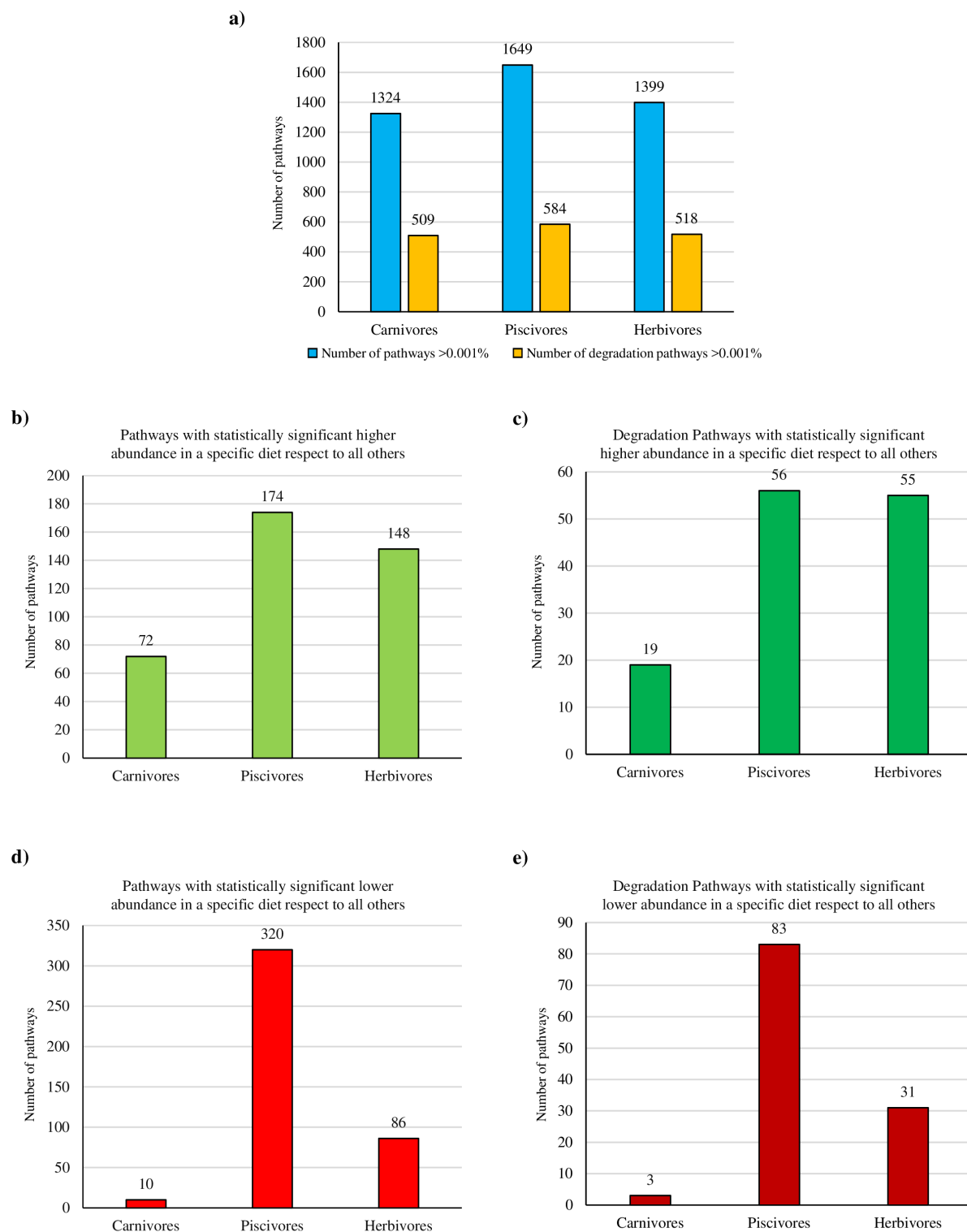


Figure 4

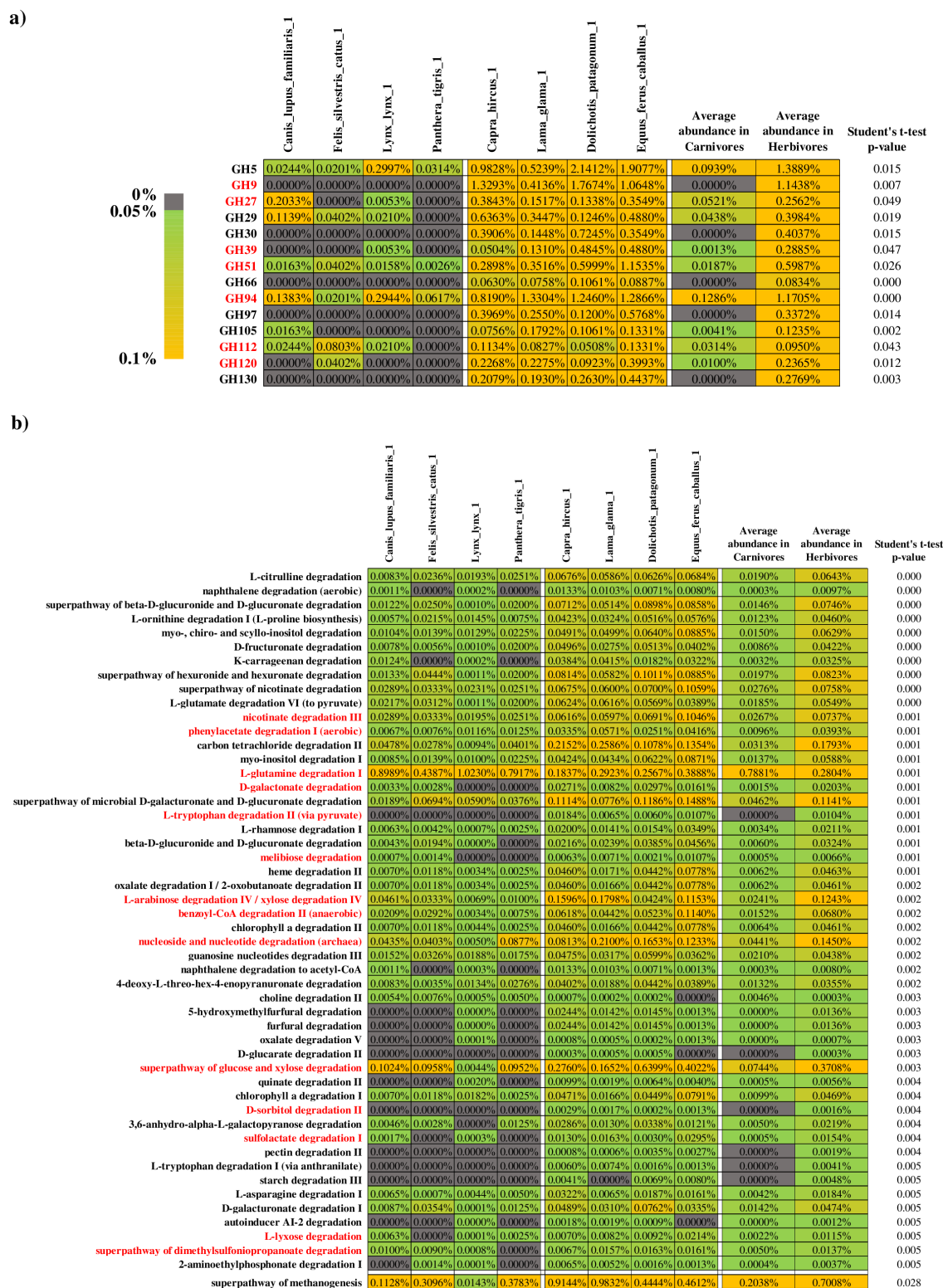


Figure 5

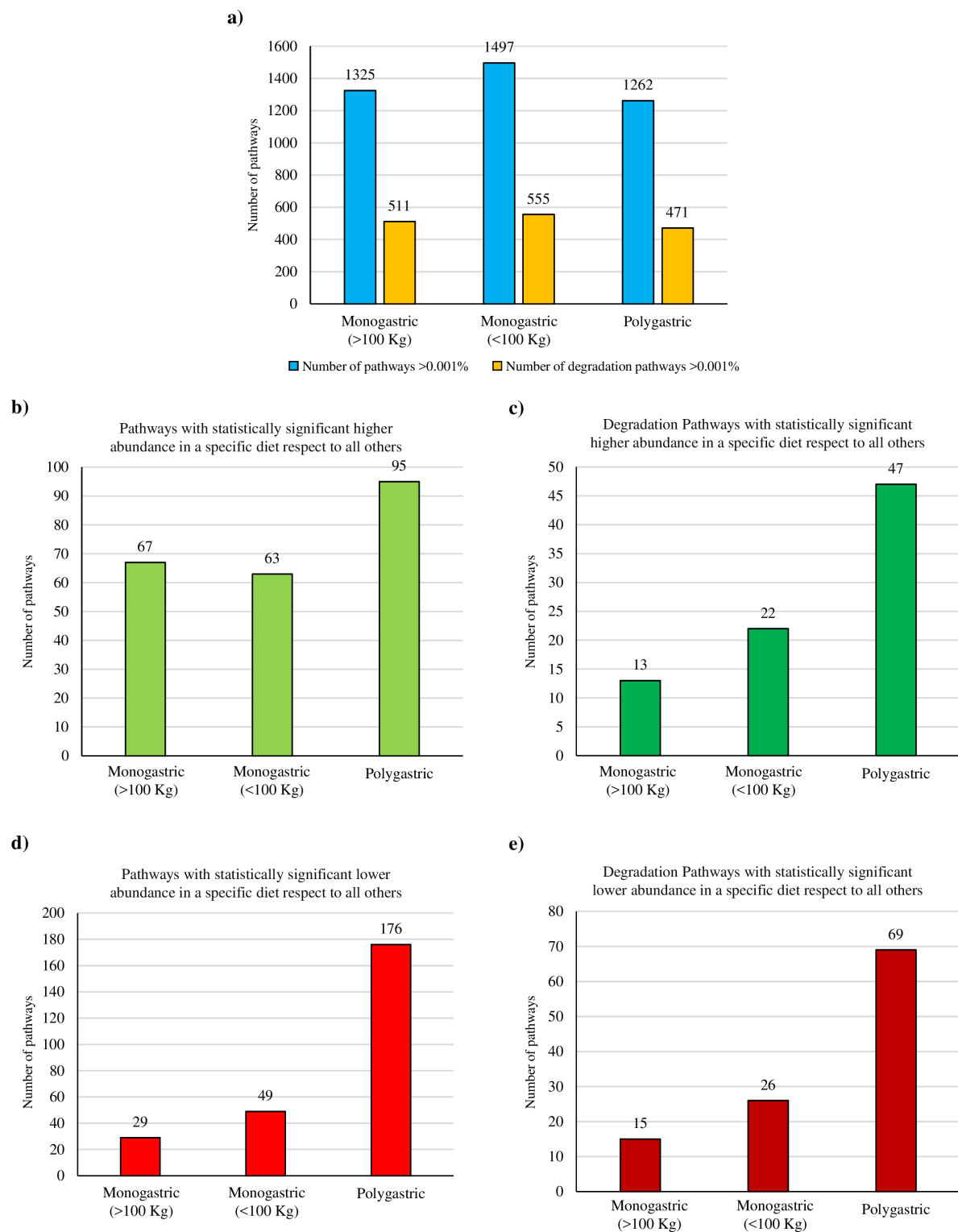


Figure 6