

Title	Multi-omics approaches to decipher the impact of diet and host physiology on the mammalian gut microbiome
Authors	Milani, Christian;Alessandri, Giulia;Mancabelli, Leonardo;Mangifesta, Marta;Lugli, Gabriele Andrea;Viappiani, Alice;Longhi, Giulia;Anzalone, Rosaria;Duranti, Sabrina;Turroni, Francesca;Ossiprandi, Maria Cristina;van Sinderen, Douwe
Publication date	2020-11-10
Original Citation	Milani, C., Alessandri, G., Mancabelli, L., Mangifesta, M., Lugli, G. A., Viappiani, A., Longhi, G., Anzalone, R., Duranti, S., Turroni, F., Ossiprandi, M. C. and van Sinderen, D. (2020) 'Multi-omics approaches to decipher the impact of diet and host physiology on the mammalian gut microbiome', Applied and Environmental Microbiology, 86(23), e01864-20 (21pp). doi: 10.1128/AEM.01864-20
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1128/AEM.01864-20
Rights	Open access - http://creativecommons.org/licenses/by/4.0/© 2020, American Society for Microbiology.
Download date	2025-08-04 00:18:57
Item downloaded from	https://hdl.handle.net/10468/12479



University College Cork, Ireland Coláiste na hOllscoile Corcaigh AEM Accepted Manuscript Posted Online 18 September 2020 Appl. Environ. Microbiol. doi:10.1128/AEM.01864-20 Copyright © 2020 American Society for Microbiology. All Rights Reserved.

1	Deciphering the impact of diet and host physiology on the mammalian gut microbiome by
2	multi-omics approaches
3	
4	Running title: the mammalian gut microbiota and diet
5	Key words: microbiota, metagenomics, mammals, metatranscriptomics, diet, physiology
6	
7	
8	Christian Milani ^{2,4*} , Giulia Alessandri ^{1*} , Leonardo Mancabelli ² , Marta Mangifesta ² , Gabriele
9	Andrea Lugli ² , Alice Viappiani ³ , Giulia Longhi ³ , Rosaria Anzalone ³ , Sabrina Duranti ² , Francesca
10	Turroni ^{2,4} , Maria Cristina Ossiprandi ^{1,4} , Douwe van Sinderen ⁵ and Marco Ventura ^{2,4}
11	*These authors contributed equally. Author order was defined based on seniority.
12	
13	
14	¹ Department of Veterinary Medical Science, University of Parma, Parma, Italy;
15	² Laboratory of Probiogenomics, Department of Chemistry, Life Sciences, and Environmental
16	Sustainability, University of Parma, Parma, Italy;
17	³ GenProbio srl, Parma, Italy;
18	⁴ Microbiome Research Hub, University of Parma, Parma, Italy;
19	⁵ APC Microbiome Institute and School of Microbiology, Bioscience Institute, National University
20	of Ireland, Cork, Ireland
21	
22	Correspondence. Mailing address for Marco Ventura Laboratory of Probiogenomics, Department of
23	Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parco Area delle
24	Scienze 11a, 43124 Parma, Italy. Phone: ++39-521-905666. Fax: ++39-521-905604. E-mail:
25	marco.ventura@unipr.it

26

Abstract

In recent years various studies have demonstrated that the gut microbiota influences host 27 metabolism. However, these studies were primarily focused on a single or a limited range of host 28 29 species, thus preventing a full exploration of possible taxonomic and functional adaptations by gut microbiota members as a result of host-microbe co-evolution events. In the current study, the 30 microbial taxonomic profiles of 250 fecal samples, corresponding to 77 host species that cover the 31 32 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 33 the fecal microbiomes of 24 mammals and subsequent statistical analyses were performed to assess 34 35 the impact of host diet and corresponding physiology of the digestive system on gut microbiota composition and functionality. Functional data was confirmed and extended through 36 metatranscriptome assessment of gut microbial populations of eight animals, thus providing insights 37 38 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 39 40 gut microbiota have adopted to maximize energy extraction from the host's diet.

41 Importance

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

2

51 both metabolic features and transcriptional responses of the intestinal bacterial community based on

52 different diets.

Applied and Environ<u>mental</u>

Microbiology

53 Introduction

The functional roles exerted by the mammalian gut microbiota have in recent years been scrutinized 54 by a range of studies focusing on multiple aspects of host biology, including the immune, digestive 55 and nervous systems (1-4). In this regard, gut microbiota composition has been shown to be 56 influenced by host genetics (5-11) as well as environmental factors that are linked to host lifestyle 57 and diet (7, 10, 12-14). Microbe-host interactions are the result of intricate adaptive occurrences, 58 59 through a process known as host-microbe co-evolution, being responsible for the adaptation of mammals to new environmental niches and having contributed to their dispersal and current global 60 distribution (5, 15). Among multiple factors, diet, host evolutionary history and host physiology are 61 62 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 63 microbial communities have revealed associations between the composition of the gut ecosystem 64 65 and host diet, even among phylogenetically un-related hosts (5, 18), and supported the notion that diet contributes to the microbiome plasticity by selecting particular metabolic activities to allow 66 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 microbiomes harbour genes which encode enzymatic activities involved in the breakdown of 69 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, 21). In concert with diet, host phylogeny and physiology have been proposed as other crucial 72 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). Indeed, despite the inter-individual fluctuations of gut microbiomes and the possible rapid changes 76 77 in response to diet and environment, it has been demonstrated that the mammalian gut microbiota

4

78 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, 79 have an important role in shaping the intestinal microbial community across mammals (7). 80 81 However, this conserved pattern of host-microbe phylosymbiosis seems to be restricted to mammals. Indeed, meta-analysis performed on fecal samples of various bird, fish, reptile or 82 amphibian species failed to report the same strict correlation (7, 16). Altogether, these findings 83 84 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 85 gut microbiota and their associated host diet and/or digestive system (5, 7, 9, 10). Nevertheless, 86 87 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 88 microbial populations has not been fully explored. Indeed, despite the extensive number of 89 90 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 91 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 investigate transcriptional profiles of the collected samples. In this context, in order to expand our 94 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 assessed by means of metagenomics (16S rRNA microbial profiling and shotgun metagenomics) 97 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

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

Accepted Manuscript Posted Online

Applied and Environ<u>mental</u>

Microbiology

The gut microbiota biodiversity across the mammalian branch of the tree of life. We 108 performed 16S rRNA gene-based microbial profiling of 250 fecal samples corresponding to 77 109 110 mammalian species, together forming a broad coverage of the mammalian tree of life (Table S1) (Supplementary Excel File 1). Specifically, the enrolled mammalian species represent 66 111 omnivores, 63 carnivores, 115 herbivores (encompassing different sub-classes accordingly to the 112 113 physiology of their digestive tract) and 6 piscivores (Table 1). In this context, because of the difficulties in collecting multiple fecal samples from non-domesticated mammals, some of the fecal 114 samples were collected from wild animals (i.e. wolves or boars) while others were retrieved from 115 116 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 117 dolphins (three fecal samples per dolphin species) (Table S1). Illumina sequencing produced a total 118 119 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 harboured by each sample, was performed through 120 121 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 mammalian taxonomic families included in this study revealed that some herbivorous mammalian 123 124 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 *p*-value of <0.001 (Figure 1a). This observation is confirmed by average diet-based rarefaction 126 127 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 mammalian gut positively correlates with the abundance of plant-based foods in the diet (p-value < 130 131 0.001), suggestive of a major metabolic role played by bacteria in the gastrointestinal tract of

132 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 133 that we obtained revealed that polygastric herbivores, including ruminants and pseudo-ruminants 134 135 (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 136 only exception was represented by hippopotamidae that showed lower biodiversity. Notably, this 137 138 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 139 significant variation in the number of observed OTUs based on their size (Figure S1b). In detail, 140 141 '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 142 compared to that of 'heavier' (>100 Kg of average body weight) monogastric herbivores 143 144 (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 145 146 (26). For this purpose, cecum fermenters possess an enlarged cecum, which retains small food 147 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 148 149 encumbrance of a large hindgut, thus being advantageous for small animals with high ratio of food 150 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 151 152 biodiversity observed in heavier monogastric herbivores (Figure S1b).

153

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

Applied and Environmental

Microbiology

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

Furthermore, in depth analysis of the microbial taxonomic profiles reconstructed from 16S rRNA 168 169 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 peculiarly high average abundance of the genus Fusobacterium and members of the 173 Ruminococcaceae family, respectively (Figure 2b). In this context, it has previously been shown 174 175 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 fibre-based diet, since the 176 177 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 therefore represent prime targets for further genomic and functional studies. In this regard, analysis 180 181 of the herbivorous gut microbiota revealed that the *in silico* predicted genera UCG-005 and UCG-

Applied and Environ<u>mental</u>

Microbiology

182 010 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 183 body weight) is characterized by a higher number of class-specific taxa when compared to other 184 185 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 186 compared to colon fermenters, i.e. heavier monogastric animals (>100 Kg-body average weight), 187 188 and ruminants (26).

189

Co-variance of gut colonizers across the mammalian tree of life. The composition and dynamics 190 191 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 192 behaviours between members of the mammalian gut microbiota (29-31). In order to investigate such 193 194 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 195 196 were used to construct a force-driven network where attractive and repulsive forces between nodes 197 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, 198 colouring of the nodes based on modularity class analysis (resolution of 0.6) revealed the presence 199 200 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 201 202 3a). Moreover, node colouring corresponding to taxa found to be associated with specific diets (p-203 value <0.05) (Figure 2) (Figure 3b) revealed that genera more abundant in herbivores, carnivores 204 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 205 206 located near clusters associated with herbivores or carnivores, reflecting the mixed diet followed by

Applied and Environ<u>mental</u>

Microbiology

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 coloured to 214 report genera showing higher relative abundance (p-value <0.05) in either of these two dietary 215 216 groups (Table S2) (Figure 3c). Since the distance between nodes is weighted on statistically significant co-occurrence and co-exclusion interactions, this network analysis revealed that genera 217 found to be more abundant in herbivores form a tighter cluster when compared to carnivore-specific 218 219 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 supported by the higher average number of co-variances observed, as represented by node size, 223 224 between herbivore-associated genera as compared to those corresponding to carnivores (Figure 3c). 225

Functional characterization of the mammalian gut microbiota. The 16S rRNA gene-based 226 microbial profiling analysis revealed substantial differences in the taxonomic composition of the 227 228 250 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 populations, a shotgun metagenomic approach was performed for 24 fecal samples. Specifically, to 230 231 obtain a balanced analysis, fecal samples were chosen in order to be equally divided per diet

AEA

232 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 233 sites in order to limit geographical biases. Data retrieved from shotgun sequencing comprised a total 234 235 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 205,386,184 236 reads with an average of 8,557,758 reads per sample (Table S3). The obtained sequence datasets 237 238 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 239 pathways (constituting an average of >0.001 % reads of the datasets) and a higher number of 240 241 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 242 of the gut microbiota of these piscivores (further details related to data collected from piscivores 243 244 (dolphins) and their relative functional assessment are reported in Supplementary Text). Furthermore, statistical analysis revealed that carnivores possess a lower number of pathways with 245 246 differential (higher or lower) abundance when compared to other diets (Bonferroni post-hoc test p-247 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, 248 249 that the herbivore gut microbiome is enriched in carbohydrate degradation pathways when 250 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, 251 252 sucrose, starch and maltose (32-34) (Table S5), predicting that the gut microbiome has a greater 253 capacity to recover energy from a plant/vegetable-based diet. In contrast, the carnivore gut 254 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 255 256 amine principally found in meats, is known as precursor of trimethylamine (35, 36). In this context,

AEM

257 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 258 observations which suggested that the carnivore microbiome is specialized to derive energy from 259 260 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 261 impact of the metabolic pathways encoded by these mammalian intestinal microbial communities. 262

263

Differences between the gut glycobiome of carnivores and herbivores. Shotgun metagenomic 264 data were also used to reconstruct the glycobiome, i.e. the genetic repertoire responsible for 265 266 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 267 on the comparison between the glycobiome profiles of carnivores and herbivores, we performed a 268 269 Student's t-test statistical analysis. Results revealed that a large number of GH families possess 270 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-271 272 derived host glycans and their degradation products (GH20, GH33, GH92, GH101, GH123, GH125 and GH129) as well as $\alpha(1\rightarrow 4)$ linked glucose polysaccharides (GH15, GH63 and GH126) such as 273 274 the animal storage carbohydrate glycogen (Table S6). Moreover, carnivores showed higher 275 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, 276 277 herbivore data extended the above observed specialization of their microbiota toward the 278 metabolism of plant-related polysaccharides such as cellulose, xylans and galactans (GH9, GH10, 279 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 280 281 mycodextran (GH87) (Table S6).

Applied and Environ<u>mental</u>

Microbiology

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.

287

288 Metatranscriptomic analysis of Carnivores and Herbivores microbiomes. Metagenomics data provided interesting information regarding functional commitment of the gut microbiota of 289 herbivores and carnivores towards metabolism of specific dietary components. In order to evaluate 290 291 if transcriptional profiles of these microbiomes reflect such observations, we performed metatranscriptome analysis of fecal samples from four carnivores and four herbivores (Table S7), 292 which were selected in order to represent various animal genera. Sequenced metatranscriptome 293 294 datasets 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 295 296 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).

Inspection of transcriptional data revealed that the range of pathways involved in the breakdown of 298 299 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 following this diet (Table S5). Similarly, analysis of the expressed glycobiomes focusing on GH 301 families showing differential abundance in metagenomic data, evidenced that genetic members of 302 303 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 contrast, genes encoding GH20, GH33 and GH129 family enzymes, which are predicted to be 305 306 involved in degradation of host-derived glycans, showed higher transcription levels in carnivores

Applied and Environ<u>mental</u>

Microbiology

(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.

310 To further explore possible differential expression of metabolic pathways and GHs showing comparable abundance in metagenomic data collected from herbivores and carnivores, statistical 311 analyses were extended to include all profiled pathways and GHs (Figure 5a). These analyses of 312 313 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 314 degradation (Figure 5a). Among the latter, members of GH5 encompass cellulases, of GH97 include 315 316 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 317 pathways involved in the metabolism of pectin, including its metabolites 4-deoxy-L-threo-hex-4-318 319 enopyranuronate, D-galacturonate and D-fructuronate, as well as the cell wall component L-320 rhamnose showed higher expression in herbivores (Figure 5a), despite comparable abundance of 321 their corresponding genes in metagenomic datasets of carnivores. In addition, the super-pathway of 322 methanogenesis showed higher expression in herbivores (Figure 5b), possibly reflecting the major metabolic role exerted by methanogens in this class of mammals (37). 323

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.

329

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

AEM

splied and Environmental Microbioloay

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

to cover the four main classes of herbivores depicted by analysis of 16S rRNA gene microbial

monogastric herbivores (Figure 6), with a similar trend observed for degradative pathways (Figure6). A possible explanation for these results is that the higher complexity of the digestive system of

Accepted Manuscript Posted Online

AEM

polygastric herbivores requires less participation of gut microbiota in the associated catabolicprocesses when compared to the situation in monogastric mammals.

In contrast, the analysis of shotgun metagenomics data showed that the gut microbiota of lighter 359 360 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 361 lighter monogastric herbivores possess the lowest gut biodiversity among herbivores (p-value < 362 363 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 364 bacterial community of lighter monogastric mammals compensates its reduced biodiversity by 365 366 maximizing its metabolic potential when compared to heavy herbivores with a more complex digestive system. 367

In order to further explore peculiar catabolic capabilities of the enrolled classes of herbivores, a 368 369 detailed evaluation of degradative metabolic pathways enriched in a specific class (ANOVA post-370 hoc Bonferroni p-value <0.05 when compared to either of the other groups) was performed (Table 371 S10). Notably, the gut microbiota of the heavier monogastric herbivores revealed a specific 372 commitment towards degradation of glycerol and a range of aromatic compounds including plant 373 metabolites, such as 2, 3-dihydroxybenzoate, or environmental pollutants such as catechol, phenol 374 and toluene (39-41) (Table S10). In contrast, the gut microbial population of lighter monogastric 375 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, 376 377 pectin and rhamnogalacturonan along with reduction of the inorganic compound sulphate into 378 hydrogen sulphide (Table S10). This observation may suggest that the higher biodiversity of heavier 379 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 380 381 populations of lighter monogastric herbivores (Figure S1) appear more specialized to promote

Accepted Manuscript Posted Online

Applied and Environ<u>mental</u> Microbiology

AEM

382 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-383 or di-saccharides) such as D- and L-arabinose, fucose, maltose, melibiose, trehalose and xylose, this 384 385 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 386 role in performing specific metabolic tasks not only dependent on host diet but also on the 387 388 physiology of the corresponding digestive system.

Further exploration of the metabolic potential of herbivores through analysis of their glycobiome 389 revealed that the microbiome of lighter monogastric herbivores encode the highest number of GH 390 391 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 chitinase activity (associated with GH19), which participate in the hydrolysis of $(1\rightarrow 4)$ - β -linkages 393 394 between N-acetyl-D-glucosamine residues in the chitin-derived chitodextrins (GH25 and GH73), induce breakdown of 1,3- β -glucans (GH81) or encode broad spectrum β -glucosidases and β -395 396 mannosidases (GH1). In this context, all these predicted enzymatic activities may suggest a genetic specialization toward degradation of the main fungal cell wall components (42) (Table 3). 397 Moreover, three of the four GH families enriched in heavier monogastric herbivores are involved in 398 399 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 400 capability of these animals to metabolize complex plant saccharides when compared to polygastric 401 402 ruminants. Furthermore, the abundance of GH family 79, which is enriched in polygastric herbivores (by 803 % and 3981 %) when compared to lighter and heavier monogastric herbivores, 403 respectively (Table 3), is linked to the degradation of proteoglycans (such as arabinogalactan-linked 404 405 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.

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

411

412 Conclusions

A wide range of studies has suggested that diet and host physiology exert a crucial role in the 413 modulation of both the taxonomical composition and metabolic repertoire of the mammalian gut 414 415 microbiota. However, these studies did focus on specific diets and included a limited number of host species. For this reason, a precise dissection of the peculiar features that characterize the gut 416 microbiota functionality in animals with specific dietary habits and an associated digestive system 417 418 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 the intestinal microbial biodiversity but also the gut microbiota composition. In detail, 16S rRNA 422 423 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 that bacterial taxa typical of mammals following a specific diet co-occur in the gut environment 425 426 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 from a functional point of view, strengthening the idea that the gut microbiota developed to 429 430 optimize energy extraction from food. Indeed, among the herbivores, differences in the bacterial

431 biodiversity and taxonomical composition were observed when considering the physiology of their digestive system. These observations were further confirmed by comparison of the herbivore 432 intestinal metabolic repertoire, showing that differences in the physiology of the digestive system 433 434 correspond to diverse microbial metabolic capabilities. Altogether, these results suggest that mammalian gut microbiota has developed in order to achieve extensive metabolic interplay aimed 435 at maximizing energy and nutrient extraction based on specific dietary habits. However, the 436 437 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 438 this context, the piscivore group is represented only by certain species of dolphins, thus limiting the 439 440 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 441 affected by human influence and captivity. Therefore, further investigations aiming to retrieve fecal 442 443 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 444 445 features co-evolved with the host. In addition, a follow-up study aimed at collecting fecal samples 446 from different mammals at different time points may be useful to better assess whether the observed 447 differences persist over time or if they are the results of transient shifts.

449 Materials and Methods

448

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.

20

Applied and Environmental

Microbiology

455 Sample collection. A total of 250 stool samples were collected through a collaboration with several Italian zoological parks and farms. In case of aquatic mammals, sample collection was performed 456 during a routine veterinary examination through rectal swabs to avoid contamination (Table S1). 457 458 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 459 with any probiotics or drugs, such as antibiotics, during the six previous months (Table S1). In all 460 461 cases, an aliquot of each fecal sample was transferred into a fecal container with RNAlater. All samples were kept on ice and shipped to laboratory under frozen conditions where they were 462 preserved at -80 °C, until they were processed. 463

464 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 465 QIAamp DNA Stool Mini Kit following the manufacturer's extraction (Qiagen). Partial 16S rRNA 466 467 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 468 sequences were added to the partial 16S rRNA gene-specific amplicons, which were further 469 470 processed involving the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B – Illumina). Amplifications were carried out using a Verity Thermocycler 471 (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a 472 473 2200 TapeStation Instrument (Agilent Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic 474 475 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 Oubit quantification system (Life 478 Technologies, USA). Amplicons were diluted to a concentration of 4 nM, and 5 μ L 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 481 482 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 483 between 140 and 400 bp and mean sequence quality score >20 while sequences with homopolymers 484 485 >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 486 (OTUs) were defined at 100 % sequence homology using DADA2 (47); OTUs not encompassing at 487 488 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 489 the lowest possible taxonomic rank using QIIME2 (46, 48) and a reference dataset from the SILVA 490 491 database v.132 (49). Biodiversity within a given sample (alpha-diversity) was calculated 492 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). 493 494 The range of similarities is calculated between values 0 and 1. PCoA representations of betadiversity were performed using QIIME2 (46, 48). 495

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

Applied and Environmental Microbiology

AEM

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 to 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 datasets were assessed using custom scripts based on RapSearch2 software (52) and the CAZy database or the MetaCyc database (53), respectively.

510 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 511 (Qiagen, UK) in a sterile tube containing glass beads (Merck, Germany). The cells were lysed 512 513 alternating 2 minutes of stirring the mix on a Precellys 24 homogenizer (Bertin instruments, France) with 2 minutes of static cooling; this step was repeated three times. The lysed cells were centrifuged 514 at 12,000 rpm for 15 min and the upper phase was recovered. The RNA samples were purified 515 516 using the RNAesy Mini Kit (Qiagen, UK) following the manufacturer's protocol. RNA 517 concentration and purity were evaluated by a Picodrop microliter spectrophotometer (Picodrop, 518 UK).

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

Applied and Environ<u>mental</u>

Microbiology

529

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

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

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

541

Funding 542

543 This work was primarily funded by the EU Joint Programming Initiative – A Healthy Diet for a 544 Healthy Life (JPI HDHL, http://www.healthydietforhealthylife.eu/) to DvS (in conjunction with Science Foundation Ireland [SFI], Grant number 15/JP-HDHL/3280) and to MV (in conjunction 545 546 with MIUR, Italy). The study is supported by Fondazione Cariparma, under TeachInParma Project (DV). GA is supported by Fondazione Cariparma, Parma, Italy. We furthermore thank GenProbio 547 srl for financial support of the Laboratory of Probiogenomics. 548

549

550 Acknowledgements

This research benefited from the HPC (High Performance Computing) facility of the University of 551 Parma, Italy. The authors declare that they have no competing interests. We thank 'Fondazione 552 553 Bioparco di Roma' (Viale del Giardino Zoologico 20, 00197, Rome, Italy), 'Zoomarine' (Via dei

Applied and Environmental Microbiology

AEM

Romagnoli, 00040 Torvaianica, Pomezia, Rimini, Italy), 'Giardino Zoologico di Pistoia' (Via Pieve 554 a Celle, 160, 51100, Pistoia, Italy), 'Zoo Safari Ravenna' (Via dei Tre Lati 2x, 48125, Ravenna, 555 Località Mirabilandia-Savio di Ravenna, Italy), 'Centro Tutela e Ricerca Fauna Esotica e Selvatica 556 557 Monte Adone' (Via Brento, 9, 40037, Sasso Marconi, Bologna, Italy) and 'Parco Faunistico Spormaggiore' (Via Nazionale, 38010 Spormaggiore, Trento, Italy) for their support in the 558 recruitment of the samples. 559

Competing interests 560

561	The	authors	declare	that	they	have	no	competing	interest.

562 **References**

563 1. McFall-Ngai M, Hadfield MG, Bosch TC, Carey HV, Domazet-Loso T, Douglas AE, Dubilier N, Eberl G, 564 Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf 565 JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ. 566 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci U S A 567 110:3229-36. 568 2. Tremaroli V, Backhed F. 2012. Functional interactions between the gut microbiota and host 569 metabolism. Nature 489:242-9. 570 3. Round JL, Mazmanian SK. 2009. The gut microbiota shapes intestinal immune responses during 571 health and disease. Nat Rev Immunol 9:313-23. 572 4. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, Hibberd ML, Forssberg H, 573 Pettersson S. 2011. Normal gut microbiota modulates brain development and behavior. Proc Natl 574 Acad Sci U S A 108:3047-52. 575 5. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, 576 Schrenzel MD, Knight R, Gordon JI. 2008. Evolution of mammals and their gut microbes. Science 577 320:1647-51. 578 6. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, 579 Knight R, Bell JT, Spector TD, Clark AG, Ley RE. 2014. Human genetics shape the gut microbiome. 580 Cell 159:789-99. 581 7. Song SJ, Sanders JG, Delsuc F, Metcalf J, Amato K, Taylor MW, Mazel F, Lutz HL, Winker K, Graves 582 GR, Humphrey G, Gilbert JA, Hackett SJ, White KP, Skeen HR, Kurtis SM, Withrow J, Braile T, Miller 583 M, McCracken KG, Maley JM, Ezenwa VO, Williams A, Blanton JM, McKenzie VJ, Knight R. 2020. 584 Comparative Analyses of Vertebrate Gut Microbiomes Reveal Convergence between Birds and Bats. 585 mBio 11. 586 8. Goodrich JK, Davenport ER, Clark AG, Ley RE. 2017. The Relationship Between the Human Genome 587 and Microbiome Comes into View. Annu Rev Genet 51:413-433. 588 9. Gaulke CA, Arnold HK, Humphreys IR, Kembel SW, O'Dwyer JP, Sharpton TJ. 2018. Ecophylogenetics 589 Clarifies the Evolutionary Association between Mammals and Their Gut Microbiota. mBio 9. 590 10. Nishida AH, Ochman H. 2018. Rates of gut microbiome divergence in mammals. Mol Ecol 27:1884-591 1897. 592 Benson AK. 2015. Host genetic architecture and the landscape of microbiome composition: humans 11. 593 weigh in. Genome Biol 16:203. 594 Carmody RN, Gerber GK, Luevano JM, Jr., Gatti DM, Somes L, Svenson KL, Turnbaugh PJ. 2015. Diet 12. 595 dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe 17:72-84. 596 13. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma 597 Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. 2014. Diet rapidly and reproducibly alters 598 the human gut microbiome. Nature 505:559-63. 599 14. Alessandri G, Milani C, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Duranti S, Turroni F, 600 Ossigrandi MC, van Sinderen D, Ventura M. 2019. The impact of human-facilitated selection on the 601 gut microbiota of domesticated mammals. FEMS Microbiol Ecol 95. 602 15. Moran NA. 2006. Symbiosis. Curr Biol 16:R866-71. 603 Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C, Stalder G, Ley RE, Farnleitner AH. 16. 604 2019. Host diet and evolutionary history explain different aspects of gut microbiome diversity 605 among vertebrate clades. Nat Commun 10:2200. 606 17. Amato KR, J GS, Song SJ, Nute M, Metcalf JL, Thompson LR, Morton JT, Amir A, V JM, Humphrey G, 607 Gogul G, Gaffney J, A LB, G AOB, F PC, Di Fiore A, N JD, T LG, Gomez A, Kowalewski MM, R JL, Link A, 608 M LS, Tecot S, B AW, K EN, R MS, Knight R, S RL. 2019. Evolutionary trends in host physiology outweigh dietary niche in structuring primate gut microbiomes. ISME J 13:576-587. 609

Onlin	
osted	
<u> </u>	610
b	611
12	612
SC	613
2	614
ol	615
Š	616
	617
a	618
-to-	619
0	620
S	621
Ā	622
	623
	624
	625

¢

AEM

610 611	18.	Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L, Henrissat B, Knight R, Gordon JI. 2011. Diet drives convergence in gut microbiome functions across mammalian
612 613 614	19.	Kohl KD, Weiss RB, Cox J, Dale C, Dearing MD. 2014. Gut microbes of mammalian herbivores
615 616	20.	Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. 2008. Worlds within worlds: evolution of the
617	21	Puscell ID. Duchlik II. 2001. Fasters that alter rumon misrohial acalery. Science 202:1110-22
619	21.	Rossell JB, Rychilk JE. 2001. Factors that alter Fumen find oblat ecology. Science 252.1115-22.
610	22.	Polationships and Europian Efforts of Microbial Communities across Host Evolutionary History
620		PLoS Biol 14:e2000225.
621	23.	Sanders JG, Powell S, Kronauer DJ, Vasconcelos HL, Frederickson ME, Pierce NE. 2014. Stability and
622		phylogenetic correlation in gut microbiota: lessons from ants and apes. Mol Ecol 23:1268-83.
623	24.	Moeller AH, Li Y, Mpoudi Ngole E, Ahuka-Mundeke S, Lonsdorf EV, Pusey AE, Peeters M, Hahn BH,
624		Ochman H. 2014. Rapid changes in the gut microbiome during human evolution. Proc Natl Acad Sci
625		U S A 111:16431-5.
626	25.	Groussin M, Mazel F, Sanders JG, Smillie CS, Lavergne S, Thuiller W, Alm EJ. 2017. Unraveling the
627		processes shaping mammalian gut microbiomes over evolutionary time. Nat Commun 8:14319.
628	26.	Dehority BA. 2002. Gastrointestinal tracts of herbivores, particularly the ruminant: Anatomy,
629		physiology and microbial digestion of plants. Journal of Applied Animal Research 21:145-160.
630	27.	Vital M. Gao J. Rizzo M. Harrison T. Tiedie JM. 2015. Diet is a major factor governing the fecal
631		butvrate-producing community structure across Mammalia. Aves and Reptilia. ISME J 9:832-43.
632	28.	La Reau AJ, Suen G, 2018. The Ruminococci: key symbionts of the gut ecosystem. J Microbiol
633		56:199-208.
634	29.	Covte KZ, Rakoff-Nahoum S. 2019. Understanding Competition and Cooperation within the
635	-	Mammalian Gut Microbiome. Curr Biol 29:R538-R544.
636	30.	Garcia-Bayona L. Comstock LE. 2018. Bacterial antagonism in host-associated microbial
637		communities. Science 361.
638	31.	Milani C. Mangifesta M. Mancabelli L. Lugli GA. James K. Duranti S. Turroni F. Ferrario C. Ossiprandi
639	-	MC, van Sinderen D. Ventura M. 2017. Unveiling bifidobacterial biogeography across the
640		mammalian branch of the tree of life. ISME J 11:2834-2847.
641	32.	Kotake T. Yamanashi Y. Imaizumi C. Tsumurava Y. 2016. Metabolism of L-arabinose in plants. J Plant
642		Res 129:781-792.
643	33.	Milani C, Lugli GA, Duranti S, Turroni F, Mancabelli L, Ferrario C, Mangifesta M, Hevia A, Viappiani
644		A, Scholz M, Arioli S, Sanchez B, Lane J, Ward DV, Hickey R, Mora D, Segata N, Margolles A, van
645		Sinderen D, Ventura M. 2015. Bifidobacteria exhibit social behavior through carbohydrate resource
646		sharing in the gut. Sci Rep 5:15782.
647	34.	Jones SA, Jorgensen M, Chowdhury FZ, Rodgers R, Hartline J, Leatham MP, Struve C, Krogfelt KA,
648		Cohen PS, Conway T. 2008. Glycogen and maltose utilization by Escherichia coli O157:H7 in the
649		mouse intestine. Infect Immun 76:2531-40.
650	35.	Wang Z, Bergeron N, Levison BS, Li XS, Chiu S, Jia X, Koeth RA, Li L, Wu Y, Tang WHW, Krauss RM,
651		Hazen SL. 2019. Impact of chronic dietary red meat, white meat, or non-meat protein on
652		trimethylamine N-oxide metabolism and renal excretion in healthy men and women. Eur Heart J
653		40:583-594.
654	36.	Rath S, Heidrich B, Pieper DH, Vital M. 2017. Uncovering the trimethylamine-producing bacteria of
655		the human gut microbiota. Microbiome 5:54.
656	37.	Enzmann F. Maver F. Rother M. Holtmann D. 2018. Methanogens: biochemical background and
657		biotechnological applications. AMB Express 8:1.
658	38.	Al-Masaudi S, El Kaoutari A, Drula E, Redwan EM, Lombard V, Henrissat B. 2019. A metagenomics
659		investigation of carbohydrate-active enzymes along the goat and camel intestinal tract. Int
660		Microbiol 22:429-435.

661	39.	Aghapour AA, Moussavi G, Yaghmaeian K. 2013. Biological degradation of catechol in wastewater
662		using the sequencing continuous-inflow reactor (SCR). J Environ Health Sci Eng 11:3.
663	40.	Kahru A, Maloverjan A, Sillak H, Pollumaa L. 2002. The toxicity and fate of phenolic pollutants in the
664		contaminated soils associated with the oil-shale industry. Environ Sci Pollut Res Int Spec No 1:27-
665		33.
666	41.	Kim HJ, Choi SW, Inyang HI. 2008. Catalytic oxidation of toluene in contaminant emission control
667		systems using Mn-Ce/gamma-Al2O3. Environ Technol 29:559-69.
668	42.	Adams DJ. 2004. Fungal cell wall chitinases and glucanases. Microbiology 150:2029-35.
669	43.	Nothnagel EA. 1997. Proteoglycans and related components in plant cells. Int Rev Cytol 174:195-
670		291.
671	44.	Du H, Clarke AE, Bacic A. 1996. Arabinogalactan-proteins: A class of extracellular matrix
672		proteoglycans involved in plant growth and development. Trends in Cell Biology 6:411-414.
673	45.	Milani C, Hevia A, Foroni E, Duranti S, Turroni F, Lugli GA, Sanchez B, Martin R, Gueimonde M, van
674		Sinderen D, Margolles A, Ventura M. 2013. Assessing the fecal microbiota: an optimized ion torrent
675		16S rRNA gene-based analysis protocol. PLoS One 8:e68739.
676	46.	Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG,
677		Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald
678		D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J,
679		Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community
680		sequencing data. Nat Methods 7:335-6.
681	47.	Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-
682		resolution sample inference from Illumina amplicon data. Nat Methods 13:581-3.
683	48.	Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J.
684		2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's g2-
685		feature-classifier plugin. Microbiome 6:90.
686	49.	Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO. 2013. The SILVA
687		ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic
688		Acids Res 41:D590-6.
689	50.	Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial
690		communities. Appl Environ Microbiol 71:8228-35.
691	51.	Milani C, Casey E, Lugli GA, Moore R, Kaczorowska J, Feehily C, Mangifesta M, Mancabelli L, Duranti
692		S, Turroni F, Bottacini F, Mahony J, Cotter PD, McAuliffe FM, van Sinderen D, Ventura M. 2018.
693		Tracing mother-infant transmission of bacteriophages by means of a novel analytical tool for
694		shotgun metagenomic datasets: METAnnotatorX. Microbiome 6:145.
695	52.	Zhao Y, Tang H, Ye Y. 2012. RAPSearch2: a fast and memory-efficient protein similarity search tool
696		for next-generation sequencing data. Bioinformatics 28:125-6.
697	53.	Caspi R, Altman T, Dreher K, Fulcher CA, Subhraveti P, Keseler IM, Kothari A, Krummenacker M,
698		Latendresse M, Mueller LA, Ong Q, Paley S, Pujar A, Shearer AG, Travers M, Weerasinghe D, Zhang
699		P, Karp PD. 2012. The MetaCyc database of metabolic pathways and enzymes and the BioCyc
700		collection of pathway/genome databases. Nucleic Acids Res 40:D742-53.
701	54.	Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform.
702		Bioinformatics 25:1754-60.

704

Applied and Environmental Microbiology

Tables 705

Table 1: list of mammals whose fecal samples were collected for this study, including the number 706 707

of sampled individuals per mammalian species and their diets.

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

<	
\leq	
7	

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

Downloaded from http://aem.asm.org/ on December 11, 2020 at IRIS

\geq	
\triangleleft	

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%

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

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

721 groups.

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%	

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

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

725 groups.

726 Figure legends

727

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

733

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

740

Applied and Environ<u>mental</u>l

Microbiology

Figure 3. Co-variance force-driven network of genera profiled with relative abundance of >5 % in 741 742 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), Collinsella (24), Comamonas (25), Corynebacterium 1 (26), Cutibacterium (27), Dialister (28), 754 755 Enterococcus (29), Epulopiscium (30), Erysipelotrichaceae UCG-002 (31), Erysipelotrichaceae UCG-004 (32), Escherichia-Shigella (33), Eubacterium coprostanligenes group (Ruminococcaceae 756 family) (34), Faecalibacterium (35), Faecalibaculum (36), Family XIII AD3011 group 757 758 (Clostridiales order) (37), Fibrobacter (38), Flavobacterium (39), Fusobacterium (40), Helicobacter (41), Ignatzschineria (42), Lachnospira (43), Lactobacillus (44), Lysinibacillus (45), 759 Megamonas (46), Megasphaera (47), Myoides (48), Paenibacillus (49), Pedobacter (50), 760 761 Peptoniphilus (51), Peptostreptococcus (52), Photobacterium (53), Prevotella 2 (54), Prevotella 7 (55), Prevotella 9 (56), Prevotellaceae UCG-001 (57), Prevotellaceae UCG-003 (58), Pseudomonas 762 (59), Psychrobacter (60), Rikenellaceae RC9 group (61), Ruminiclostridium 6 (62), 763 764 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 Ricketsiales 770 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 771 772 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 (98), U. m. of Enterobacteriaceae family (99), U. m. of Erysipelotrichaceae family (100), U. m. of 775 776 Eukaryota kingdom (101), U. m. of F082 family (102), U. m. of Firmicutes phylum (103), U. m. of

Applied and Environmental

Microbiology

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

785

786 Figure 4. Metabolic pathways prediction in Carnivores, Piscivores and Herbivores. Panel a shows the number of pathways detected with abundance >0.001 %. Panels b and c report the sum of the 787 number of all pathways and degradative pathways, respectively, that showed a significantly higher 788 789 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 of the number of all pathways and degradative pathways, respectively, with significantly lower 791 792 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 different abundance in carnivores and herbivores. GHs in red show similar abundance in the 796 metagenomes of carnivores and herbivores. Panel b reports the transcriptional abundance (as a 797 798 proportion of all predicted metabolic pathways) of degradation pathways with statistically different abundance in carnivores and herbivores. Pathways in red displayed similar abundance in the 799 800 metagenomes of carnivores and herbivores.

801

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











- Escherichia-Shigella
- U. m. of Prevotellaceae family
- U. m. of Clostridiaceae 1 family
- Ruminococcaceae UCG-005
- Ruminococcaceae UCG-014
- Cetobacterium

U. m. of Muribaculaceae family

Bacteroides

Acinetobacter

Photobacterium

Enterococcus

Sarcina

- Fusobacterium
 - U. m. of Peptostreptococcaceae family
 - U. m. of Pasteurellaceae family
 - U. m. of Ruminococcaceae family
 - Christensenellaceae R-7 group
 - Others (below 10%)

b)

	2		>	
1	1	1	Ī	
<	<	1	ſ	

Figure	2
riguit	-

Phylum	Genus	Carnivores	Herbivores	Omnivores	Piscivores	P-valu
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









Pathways with statistically significant lower

abundance in a specific diet respect to all others

320

Piscivores

86

Herbivores



e)

Degradation Pathways with statistically significant lower abundance in a specific diet respect to all others



Downloaded from http://aem.asm.org/ on December 11, 2020 at IRIS

d)

350

300

250

200

150

100

50

0

10

Carnivores

Number of pathways

Figure 4

a)

a)	CH15 0.0244% of GH15	Felis_silvestris_catus_1	Lynx_lynx_1 0.2362.0	*71000	Capra_hircus_1	8% 0.52	Lama_glama_l 5 <u>%653</u>	Dolichotis_patagonum_1	Equus_ferus_caballus_1	Avera abundar Carniv 0.093	nge Av nce in abund ores Her 9% 1.3	erage lance in S bivores 889%	tudent's t-test p-value 0.015
0%	GH9 0.0000% (GH27 0.2033% (0.0000%	0.0000% 0.0053%	0.0000%	6 1.329 6 0.384	3% 0.41 3% 0.15	136% 1 517% 0	.7674% 1	0.3549%	0.000	0% 1.1 1% 0.2	438% 562%	0.007 0.049
0.05%	GH29 0.1139% (0.0402%	0.0210%	0.0000%	6 0.636	3% 0.34	47% 0	.1246% (0.4880%	0.043	8% 0.3	984%	0.019
	GH30 0.0000% (GH39 0.0000% (0.0000%	0.0000% 0.0053%	0.0000%	6 0.390 6 0.050	6% 0.14 4% 0.13	148% 0 310% 0	.7245% (.4845% ().3549%).4880%	0.000	0% 0.4 3% 0.2	037% 885%	0.015 0.047
	GH51 0.0163% (0.0402%	0.0158%	0.0026%	6 0.289	8% 0.35	516% 0	.5999% 1	.1535%	0.018	7% 0.5	987%	0.026
	GH94 0.1383% (0.0201%	0.0000% 0.2944%	0.0617%	6 0.005 6 0.819	0% 0.0. 0% 1.33	304% 1	.1081% (.2866%	0.000	6% 0.0 6% 1.1	834% 705%	0.000
	GH97 0.0000% (GH105 0.0163% (0.0000%	0.0000% 0.0000%	0.0000%	6 0.396	9% 0.25 6% 0.13	550% 0 792% 0	.1200% (.1061% ().5768%).1331%	0.000	0% 0.3 1% 0.1	372% 235%	0.014 0.002
0.1%	GH112 0.0244% (0.0803%	0.0210%	0.0000%	6 0.073 6 0.113	4% 0.08	327% 0	.0508% (0.1331%	0.031	4% 0.0	950%	0.043
0.1 /0	GH120 0.0000% (GH130 0.0000% (0.0402%	0.0000% 0.0000%	0.0000%	6 0.226 6 0.207	8% 0.22 9% 0.19	275% 0 930% 0	.0923% ().3993%).4437%	0.010	0% 0.2 0% 0.2	365% 769%	0.012 0.003
b)			upus_familiaris_1	lvestris_catus_1	'nx_1	a_tigris_1	hircus_1	țlama_1	tis_patagonum_1	ferus_caballus_1			
			anis_l	elis_sil	yllx_J	anther	apra_	ama_g	olicho	f_sunp	Average abundance in	Average abundance	in Student's t-test
	L-citrulline des	radation	0.0083%	ية 0.0236%	고 0.0193%	<u>م</u> 0.0251%	0.0676	고 % 0.05869	م 0.0626%	ي 0.0684%	Carnivores	Herbivore 0.0643%	s p-value
superpathway of beta-D-alucuron	naphthalene degradation	(aerobic)	0.0011%	0.0000%	0.0002%	0.0000%	0.0133	% 0.0103	60.0071%	0.0080%	0.0003%	0.0097%	0.000
L-ornithine deg	gradation I (L-proline bio	synthesis)	0.0057%	0.0215%	0.0145%	0.0075%	0.0423	% 0.0324	% 0.0516%	0.0576%	0.0123%	0.0460%	0.000
туо-, сп	D-fructuronate deg	gradation gradation	0.0104%	0.0139%	0.0129% 0.0010%	0.0225%	0.0491	% 0.0499% % 0.0275%	6 0.0640% 6 0.0513%	0.0885% 0.0402%	0.0086%	0.0829%	0.000
superpathway of hexu	K-carrageenan des ronide and hexuronate des	gradation gradation	0.0124%	0.0000%	0.0002% 0.0011%	0.0000%	0.0384	% 0.0415% % 0.0582%	% 0.0182% % 0.1011%	0.0322% 0.0885%	0.0032% 0.0197%	0.0325% 0.0823%	0.000
supe: L-glutz	rpathway of nicotinate deg amate degradation VI (to	gradation ovruvate)	0.0289%	0.0333%	0.0231% 0.0011%	0.0251%	0.0675	% 0.0600% % 0.0616%	60.0700% 0.0569%	0.1059%	0.0276%	0.0758%	0.000
- .	nicotinate degra	dation III	0.0289%	0.0333%	0.0195%	0.0251%	0.0616	% 0.05979	% 0.0691%	0.1046%	0.0267%	0.0737%	0.001
c C	arbon tetrachloride degra	dation II	0.0478%	0.0278%	0.0094%	0.0401%	0.0555	% 0.2586%	6.02517 60.1078%	0.1354%	0.0313%	0.1793%	0.001
	myo-mositol degr L-glutamine degr	adation I adation I	0.0085%	0.0139% 0.4387%	0.0100% 1.0230%	0.0225% 0.7917%	0.0424	% 0.0434 % 0.2923	6.0622% 0.2567%	0.0871% 0.3888%	0.0137%	0.0588%	0.001
superpathway of microbial D-galacturon	D-galactonate des ate and D-glucuronate des	gradation gradation	0.0033%	0.0028% 0.0694%	0.0000% 0.0590%	0.0000%	0.0271 0.1114	% 0.00829 % 0.07769	6 0.0297% 0.1186%	0.0161% 0.1488%	0.0015% 0.0462%	0.0203% 0.1141%	0.001 0.001
L-trypto	phan degradation II (via) L-rhamnose degr	<mark>pyruvate)</mark> adation I	0.0000%	0.0000%	0.0000%	0.0000%	0.0184	% 0.0065% % 0.0141%	6 0.0060% 0.0154%	0.0107%	0.0000%	0.0104%	0.001
beta-D-glucuron	ide and D-glucuronate deg	gradation	0.0043%	0.0194%	0.0000%	0.0000%	0.0216	% 0.02399	% 0.0385%	0.0456%	0.0060%	0.0324%	0.001
	heme degra	idation II	0.0070%	0.0118%	0.0034%	0.0025%	0.0460	% 0.01719	6.00217 6 0.0442%	0.0778%	0.0062%	0.0463%	0.001
oxalate degradatio L-arabinose degr	n 1 / 2-oxobutanoate degra radation IV / xylose degra	dation II	0.0070%	0.0118%	0.0034%	0.0025%	0.0460	% 0.0166 % 0.1798	0.0442%	0.0778%	0.0062%	0.0461%	0.002
benz	oyl-CoA degradation II (a chlorophyll a degra	naerobic) adation II	0.0209%	0.0292% 0.0118%	0.0034% 0.0044%	0.0075%	0.0618	% 0.04429 % 0.01669	% 0.0523% % 0.0442%	0.1140% 0.0778%	0.0152%	0.0680%	0.002 0.002
nucleoside an gu:	d nucleotide degradation anosine nucleotides degra	(archaea) dation III	0.0435%	0.0403%	0.0050% 0.0188%	0.0877% 0.0175%	0.0813	% 0.2100 % 0.0317	% 0.1653% % 0.0599%	0.1233%	0.0441%	0.1450%	0.002 0.002
napl 4-deoxy-L-three-h	nthalene degradation to ac ex-4-enopyranuronate dee	etyl-CoA	0.0011%	0.0000%	0.0003%	0.0000%	0.0133	% 0.0103%	% 0.0071% % 0.0442%	0.0013%	0.0003%	0.0080%	0.002
+ucoxy-1)-till to-1	choline degra	idation II	0.0054%	0.0076%	0.0005%	0.0050%	0.0007	% 0.0002	% 0.0002%	0.0000%	0.0046%	0.0003%	0.002
5-1	furfural des	gradation gradation	0.0000%	0.0000%	0.0000% 0.0000%	0.0000%	0.0244	% 0.01429 % 0.01429	6 0.0145% 6 0.0145%	0.0013%	0.0000%	0.0136%	0.003
	oxalate degra D-glucarate degra	adation V adation II	0.0000%	0.0000%	0.0001% 0.0000%	0.0000%	0.0008	% 0.0005% % 0.0005%	% 0.0002% % 0.0005%	0.0013% 0.0000%	0.0000%	0.0007% 0.0003%	0.003 0.003
superpathwa	y of glucose and xylose deg quinate degra	<mark>gradation</mark> adation II	0.1024%	0.0958% 0.0000%	0.0044% 0.0020%	0.0952% 0.0000%	0.2760 0.0099	% 0.1652% % 0.0019%	<mark>% 0.6399%</mark> % 0.0064%	0.4022%	0.0744%	0.3708% 0.0056%	0.003 0.004
	chlorophyll a degr	adation I	0.0070%	0.0118%	0.0182%	0.0025%	0.0471	% 0.01669	% 0.0449% % 0.0002%	0.0791%	0.0099%	0.0469%	0.004
3,6-anhydro-alp	pha-L-galactopyranose deg	gradation	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 pectin degradation II		0.0000%	0.0000%	0.0003%	0.0000%	0.0008	% 0.0063	% 0.0030%	0.0295% 0.0027%	0.0005%	0.0154%	0.004	
L-tryptoph	an degradation I (via antl starch degra	ranilate) dation III	0.0000%	0.0000%	0.0000% 0.0000%	0.0000%	0.0060	% 0.00749 % 0.00009	% 0.0016% % 0.0069%	0.0013% 0.0080%	0.0000%	0.0041% 0.0048%	0.005
	L-asparagine degr D-galacturonate degr	adation I adation I	0.0065%	0.0007%	0.0044% 0.0001%	0.0050%	0.0322	% 0.0065% % 0.0310%	% 0.0187% % 0.0762%	0.0161%	0.0042%	0.0184%	0.005
	autoinducer AI-2 deg	gradation	0.0000%	0.0000%	0.0000%	0.0000%	0.0018	% 0.0019%	% 0.0009%	0.0000%	0.0000%	0.0012%	0.005
superpathway of dimet	hylsulfoniopropanoate deg	gradation	0.0100%	0.0090%	0.0008%	0.0000%	0.0067	% 0.0157%	% 0.0163%	0.0161%	0.0050%	0.0137%	0.005
2-am	superpathway of metha	adation I	0.1128%	0.0014%	0.0001%	0.3783%	0.0065	% 0.0052	0.0016%	0.0013%	0.0004%	0.0037%	0.005

Figure 5



c)



Pathways with statistically significant lower

abundance in a specific diet respect to all others

49

Monogastric

(<100 Kg)

Degradation Pathways with statistically significant higher abundance in a specific diet respect to all others 47 45 40 35 30



Downloaded from http://aem.asm.org/ on December 11, 2020 at IRIS

e)

176

Polygastric

Degradation Pathways with statistically significant lower abundance in a specific diet respect to all others





d)

200

180

160

140

120

100

80

60 40

20

0

29

Monogastric

(>100 Kg)

Number of pathways

Accepted Manuscript Posted Online