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ORIGINAL ARTICLE

Diet is a major factor governing the fecal butyrate-producing community structure across *Mammalia*, *Aves* and *Reptilia*

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Butyrate-producing bacteria have an important role in maintaining host health. They are well studied in human and medically associated animal models; however, much less is known for other *Vertebrata*. We investigated the butyrate-producing community in hindgut-fermenting *Mammalia* (*n* = 38), *Aves* (*n* = 8) and *Reptilia* (*n* = 8) using a gene-targeted pyrosequencing approach of the terminal genes of the main butyrate-synthesis pathways, namely butyryl-CoA:acetate CoA-transferase (*but*) and butyrate kinase (*buk*). Most animals exhibit high gene abundances, and clear diet-specific signatures were detected with *but* genes significantly enriched in omnivores and herbivores compared with carnivores. *But* dominated the butyrate-producing community in these two groups, whereas *buk* was more abundant in many carnivorous animals. Clustering of protein sequences (5% cutoff) of the combined communities (*but* and *buk*) placed carnivores apart from other diet groups, except for noncarnivorous *Carnivora*, which clustered together with carnivores. The majority of clusters (*but:* 5141 and *buk:* 2924) did not show close relation to any reference sequences from public databases (identity <90%) demonstrating a large 'unknown diversity'. Each diet group had abundant signature taxa, where *buk* genes linked to *Clostridium perfringens* dominated in carnivores and *but* genes associated with *Ruminococcaceae bacterium* D16 were specific for herbivores and omnivores. Whereas *16S rRNA* gene analysis showed similar overall patterns, it was unable to reveal communities at the same depth and resolution as the functional gene-targeted approach. This study demonstrates that butyrate producers are abundant across vertebrates exhibiting great functional redundancy and that diet is the primary determinant governing the composition of the butyrate-producing guild.
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Introduction

Many studies investigating host–microbiota interactions have demonstrated that the intestinal microbiota has a critical role in maintaining gut homeostasis and host health. A mutualistic relationship between the host and its microbiota exists, where bacteria provide essential substrates such as vitamins or short-chain fatty acids, whereas the host delivers nutrients and a stable growth environment (Nicholson *et al.*, 2012). Most investigations have been performed with humans and a few model organisms (mouse, rat and pig), but much less is known for other mammals or distinct vertebrate classes. In a pioneer study using *16S rRNA* gene sequences, Ley *et al.* (2008) found an overall relationship in mammals between the structure of the fecal microbiota, diet and host phylogeny. Whereas studies based on traditional bacterial taxonomic markers can provide first insights into host and microbiota inter-relationships, functional investigations are essential in order to understand the key processes. Several studies focused on microbial functions for specific animals (c.f. Lavery *et al.,* 2012; Tun *et al.,* 2012), and a global metagenomic screen across *Mammalia* demonstrated that the bacterial functional repertoire is primarily governed by diet and does not reflect host phylogeny (Muegge *et al.,* 2011). That study revealed that the microbiota in carnivores and herbivores are characterized by different trade-offs in amino-acid pathways and by an opposite directionality in the central phosphoenolpyruvate-pyruvate-oxaloacetate cycle. Similar differences were observed in human subjects who were exposed to diets mirroring those of carnivores or herbivores demonstrating that broad functional differences can

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be achieved with distinct nourishments even in the same host indicating that direct host-specific selection processes are limited (at least in humans) (David *et al.*, 2013).

The divergence between overall bacterial function and host phylogeny raises the question whether specific physiological processes of the host that are linked to certain microbial functions, such as components of the immune system or energy requirements of the colonic epithelium, evolved with diet. If this is not the case and those processes are conserved throughout hosts, or coevolved with certain phylogenetic groups (for example, all mammals), we would expect functional redundancy of the microbiota that is adapted to the individual diets. In this context, the production of butyrate is of specific interest as it is considered essential for maintaining health in humans and usual animal models, where it serves as the main energy source for colonocytes, shows immunomodulatory effects and influences local gene expression (Hamer *et al.,* 2008; Furusawa *et al.,* 2013). Whereas butyrate is detected in feces of various vertebrates (Stevens and Hume, 1998), indicating that it serves important roles throughout this taxon, it is currently unknown how butyrate-producing communities evolved with distinct diets. In particular, carnivores are interesting as butyrate production is primarily linked to bacteria that feed on carbohydrates such as starch or xylan, converting pyruvate to butyrate using the 'acetyl-CoA pathway' (Bennett and Rudolph, 1995). However, this pathway can also be fed by amino acids and additional amino-acid-based butyrate-synthesis pathways are recognized as well (Buckel and Barker, 1974; Barker *et al.,* 1982; Gerhardt *et al.,* 2000).

The few existing studies specifically focusing on butyrate producers in different animal taxa demonstrate that butyrate-producing communities are omnipresent; however, their composition can be considerably distinct from that of humans (c.f. Eeckhaut *et al.,* 2011; Levine *et al.,* 2013). Detailed investigations on these important functional communities across vertebrates are missing and it is currently unknown how they have adapted to distinct hosts.

Materials and methods

Samples and DNA extraction

An overview of all collected fecal samples is given in Table 1. The majority of samples were derived from Potter Park Zoo (Lansing, MI, USA), except for dogs (private), giant panda (Ningbo, China), guinea-pig, mouse, rat (commercial pet-store) and grizzly bear (Detroit Zoo, Detroit, MI, USA). One sample per animal was collected. Only hindgut fermenters were included in the analysis. Foregut fermenters were specifically excluded to avoid influences of their distinct gut composition on the structure of butyrate-producing communities that might bias diet-related signals. All samples were collected within 24 h after defecation and were stored at −80 °C. Animals were categorized into three distinct diet groups based on feeding protocols provided by individual sources (Supplementary Table S2). Categorization into carnivores and herbivores was based on the animal's predominant diet, and some taxa in those groups were additionally fed minor amounts of substances that were not strictly associated with the corresponding diet. Zoo animals were fed diets researched to be appropriate for the taxon and used by most US zoos. DNA extraction was performed using the PowerSoil kit (MoBio, Jefferson City, MO, USA) according to the manufacturer.

Amplification of functional genes

Primers used were described previously (Vital *et al.,* 2013). Amplification of DNA samples was achieved with the AccuPrime PCR system (Life Technologies, Grand Island, NY, USA), where 5 ng of template DNA was combined with all individual forward and reverse primers containing the same barcode (total final concentration of 0.4 μM). The PCR protocol (35 cycles) was carried out according to the manufacturer, with annealing temperatures and $MgSO_4$ concentrations of 51 °C, 3 mM and 48 °C, 2 mM for butyryl-CoA:acetate CoA-transferase (*but*) and butyrate kinase (*buk*), respectively. Harvesting of target bands and re-amplification are described in (Vital *et al.,* 2013). Sequencing was performed on the 454 FLX system at the Utah State University (Logan, UT, USA) using the Lib-A kit according to the manufacturer (454 Life Sciences, Branford, CT, USA).

Functional genes' data analysis

To estimate gene abundances of *but* and *buk* (as percentage of total bacteria exhibiting the gene), target bands after the first PCR amplification were categorized into six distinct brightness groups (5: above 20%. 4: 10%–20%, 3: 4%–10%, 2: 1%–4%, 1: below 1% and 0: not detectable; category 1 was omitted for *but* gene analysis because of a higher detection threshold compared with *buk*) based on standard curves. The following concentrations from reference organisms (*but:* mix of *Roseburia inulinivorans/Faecalibacterium prausnitzii* and *buk: Clostridium perfringens*) were used to establish standard curves: 5 ng (100%), 2 ng (40%), 1 ng (20%), 0.5 ng (10%), 0.2 ng (4%), 0.1 ng (2%), 0.05 ng (1%), 0.02 ng (0.4%) and 0.01 ng (0.2%). Band brightness was measured using the software imageJ (http://imagej.nih.gov/ij/). Two independent experiments were performed and average values were used as the final result for statistical analyses. *16S rRNA* was included as an amplification control (PCR conditions and primers as presented in Kozich *et al.* (2013) but increase of cycle numbers to 35). This method was used as qPCR analysis was not possible for quantification of these genes because of the

Table 1 Diet and taxonomy of the 54 samples included in the study

SampleID	*Diet*	*Class*	*Order*	*Family*	*Genuse/species*
BaldEagle	C	Aves	Accipitriformes	Accipitridae	Haliaeetus leucocephalus
Duck	H	Aves	Anseriformes	Anatidae	Anseranser domestic
Mousebird	H	Aves	Coliiformes	Coliidae	Urocolius macrourus
Hawk	C	Aves	Falconiformes	Accipitridae	Buteo jamaicensis
Peafowl	O	Aves	Galliformes	Phasianidae	Pavo cristatus
KingVulture	C	Aves	Incertaesedis	Cathartidae	Sarcoramphus papa
Penguin	C	Aves	Sphenisciformes	Spheniscidae	Spheniscus magellanicus
Ostrich	O	Aves	Struthioniformes	Struthionidae	Struthio S. camelus
Tenrec1	C	Mammalia	Afrosoricida	Tenrecidae	Echinops telfairi
Tenrec2	C	Mammalia	Afrosoricida	Tenrecidae	Echinops telfairi
GuineaHog	H	Mammalia	Artiodactyla	Suidae	Sus scrofa scrofa
Dog1	O	Mammalia	Carnivora	Canidae	Canis lupus
Dog2	O	Mammalia	Carnivora	Canidae	Canis lupus
ArcticFox1	C	Mammalia	Carnivora	Canidae	Alopex lagopus
ArcticFox2	C	Mammalia	Carnivora	Canidae	Alopex lagopus
AfricanLion	C	Mammalia	Carnivora	Felidae	Panthera Heol krugeri
Tiger	C	Mammalia	Carnivora	Felidae	Panthera tigris
Meerkat	O	Mammalia	Carnivora	Herpestidae	Suricata suricatta
Ferret	C	Mammalia	Carnivora	Mustelidae	Mustela putorius furo
RiverOtter	C	Mammalia	Carnivora	Mustelidae	Lontra canadensis
RedPanda	H	Mammalia	Carnivora	Ursidae	Ailurus fulgens fulgens
GrizzlyBear	O	Mammalia	Carnivora	Ursidae	Ursus arctos
GiantPanda1	H	Mammalia	Carnivora	Ursidae	Ailuropoda melanoleuca
GiantPanda2	H	Mammalia	Carnivora	Ursidae	Ailuropoda melanoleuca
Binturong	O	Mammalia	Carnivora	Viverridae	Arctictis binturong
Armadillo	O	Mammalia	Cingulata	Dasypodidae	Tolypeutes matacus
Opossum	O	Mammalia	Didelphimorphia	Didelphidae	Didelphis virginiana
Rabbit1	H	Mammalia	Lagomorpha	Leporidae	Oryctolagus cuniculus
Rabbit2	H	Mammalia	Lagomorpha	Leporidae	Oryctolagus cuniculus
Donkey	H	Mammalia	Perissodactyla	Equidae	Equus asinus asinus
CottontopTamarin	O	Mammalia	Primates	Callitrichidae	Sagulnus oedipus
GoldenLionTamarin	O	Mammalia	Primates	Callitrichidae	Leontopithecus rosalia
Human1	O	Mammalia	Primates	Hominidae	Homo sapiens
Human2	O	Mammalia	Primates	Hominidae	Homo sapiens
Mandril	O	Mammalia	Primates	Cercopithecidae	Mandrillus sphinx
MongooseLemur	O	Mammalia	Primates	Lemuridae	Eulemur mongoz
RedruffeledLemur	O	Mammalia	Primates	Lemuridae	Varecia Variegatelrubra
RingtailedLemur	O	Mammalia	Primates	Lemuridae	Lemur catta
SpiderMonkey	O	Mammalia	Primates	Atelidae	Ateles fusciceps robustus
GuineaPig	H	Mammalia	Rodentia	Caviidae	Cavia porcellus
PatagonianCavy	H	Mammalia	Rodentia	Caviidae	Dolichotis patagonum
Chinchilla	H	Mammalia	Rodentia	Chinchillidae	Chinchilla brevicaudataus
Porcupine	O	Mammalia	Rodentia	Hystricidae	Hystrix cristata
Mouse	O	Mammalia	Rodentia	Muridae	Mus musculus
Rat	O	Mammalia	Rodentia	Muridae	Rattus norvegicus
Treeshrew	O	Mammalia	Scandentia	Tupaiidae	Tupaia belangeri
BeardedDragon	O	Reptilia	Squamata	Agamidae	Pogona vitticeps
MilkSnake	C	Reptilia	Squamata	Colubridae	Lampropeltis triangulum sinaloae
RatSnake	C	Reptilia	Squamata	Colubridae	Panther ophislobsoletus/spiloides[a]
Chuckwalla	H	Reptilia	Squamata	Iguanidae	Sauromalus ater
Skink	O	Reptilia	Squamata	Scincidae	Tiliqua scincoides intermedia
BoxTurtle	O	Reptilia	Testudines	Emydidae	Terrapene carolina carolina
Tortoise1	H	Reptilia	Testudines	Testudinidae	Testudo graeca traeca
Tortoise2	H	Reptilia	Testudines	Testudinidae	Testudo graeca traeca

Abbreviations: C, carnivores; H, herbivores; O, omnivores.
[a]The sample derived from either species.

formation of primer dimers as well as some nonspecific amplification (Vital *et al.*, 2013). Processing of raw reads was carried out according to Vital *et al.* (2013) with some alterations. Raw reads were processed through Ribosomal Database Project (RDP) pyrosequencing pipeline (http://rdp.cme.msu.edu) yielding 491 000 *but* and 286 761 *buk* sequences after trimming. Sequences were then frameshift-corrected with FrameBot (Wang *et al.*, 2013). During these initial pipelining steps all sequences that display ⩾93% coverage to seeding sequences from FunGene (Fish *et al.*, 2013) were included. This selection does contain many sequences of distinct functionality/substrate specificity and sequences considered as real *but*/*buk* genes (ref. seq.) form a separate clade (on a neighbor joining tree) that includes mainly well-known butyrate producers and where function of several

sequences was biochemically proven (Vital *et al.,* 2013). FunGene sequences that encode enzymes with distinct functions/substrate specificities were intentionally included here as they encompass the most closely related sequences to *but* and *buk* genes, respectively, and amplicons matching those genes were omitted from further analyses (filtered-out). Thus, amplicons were not forced to match a specific ref. seq. in order to ensure that amplicons displaying low identities to a specific ref. seq. are not originating from genes encoding distinct functionality. This exclusion criterion was previously not applied for *buk* ref. seq. (Vital *et al.,* 2013) but is included in this study because several sequences previously considered as possible *buk* are most likely of distinct functionality/substrate specificity (namely methylbutyrate kinase, Vital *et al.,* 2014). For *buk,* a few sequences linked to butyrate producers expected to exhibit that gene namely *Acidaminococcus* sp. 21, *Anaerotruncus colihominis* and *Halanaerobium praevalens* fell outside the main clade and were manually included as real *buk*. The percentage of retained sequences after the pipeline process were 66.5% and 71.1% for *but* and *buk*, respectively, excluding the samples that produced too few sequences (see below). Subsequently, chimeras were removed using UCHIME (www.drive5.com) in *de novo* mode. A very low percentage of *but* (0.2%) and *buk* (0.1%) sequences, respectively, were identified as chimeric. Remaining protein sequences were aligned to Hidden Markov Models of each individual gene (*hmmalign*), trimmed from both ends and clustered using RDP's complete-linkage-clustering applying a 5% cutoff. It should be noted that closest match assignments of amplicons during FrameBot analysis do not imply that the carrier exhibiting an amplicon/gene does necessarily fall into the same taxonomic unit associated with the closest matching ref. seq. All pipeline steps and clustering were performed using Michigan State University High Performance Computing Center (MSU HPCC). The number of clusters for individual diet groups including identity of representative sequences to a ref. seq. was calculated excluding global singletons. For ordination analysis, operational taxonomic unit tables were subsampled to equal depth for each gene (1093 sequences per sample) using the function *rrarefy* in R's (R Development Core Team, 2008) package vegan and, subsequently, combined. Some samples contained too few sequences as most (⩾97%) were filtered-out during pipeline processing (*but*: Bald Eagle, GiantPanda 1, GiantPanda 2, Penguin, Tenrec 1; *buk:* CottontopTamarin). For GrizzlyBear and Chuckwalla, only 747 *but* genes (88.5% filtered-out) and 743 *buk* genes (74% filtered-out), respectively, were retrieved. Furthermore, a few samples produced no band after re-amplification (*but*: RedPanda; *buk*: MilkSnake, Mousebird, GiantPanda 1, GiantPanda 2, Peafowl). For all those samples the corresponding gene was treated as absent during ordination analysis.

Nonmetric multidimensional scaling analysis (vegan's *metaMDS*) using Bray–Curtis dissimilarities on Hellinger-transformed data (vegan's *decostand*) was performed. Statistical analysis (permutational ANOVA) was calculated with vegan's *adonis*. For FrameBot closest match analysis, samples were normalized to equal depth (*but*: 1093 and *buk*: 1142) before analysis. Samples exhibiting values below those thresholds were not considered for analysis (the same samples as for ordination analysis were excluded). Results were separated into distinct categories based on their percent identity to a reference gene, where each category encompassed a 10% identity range. Results originating from the same genome as well as genes from closely related strains (see (Vital *et al.,* 2013)) were merged. For statistical analysis of FrameBot closest match data, the functions *mt* (test = f, R package labdsv) and vegan's *sim* (simper analysis) were used. Noncarnivorous *Carnivora* as well as taxa that exhibited <1% of the total gene communities in all samples were excluded from the analysis.

Amplification and analysis of 16S rRNA genes

Primers, barcoding strategy and amplification was performed according to (Kozich *et al.,* 2013). Samples were individually amplified, gel-extracted (QIAquick gel extraction kit; Qiagen, Valencia, CA, USA) and purified (QIAquick gel purification kit; Qiagen). All samples produced bright bands indicating that the extract was not inhibitory to amplification. Samples were pooled to equal concentrations and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at the Research Technology Support Facility at the Michigan State University. Raw paired-end reads were merged using RDP's APE (Cole *et al.,* 2014). Before read taxonomic classifications (RDP's classifier), chimeras were removed using UCHIME (www.drive5.com) using the gold database. All steps were performed using the MSU HPCC. The data were harvested for genera linked to butyrate producers according to Supplementary Table S1 (based on Vital *et al.,* 2014). Pearson correlations and corresponding statistical analyses were calculated in excel and results were visualized using cytoscape (http://www.cytoscape.org). Ordination analysis and statistics on entire communities are based on a supervised approach at the genus level (Sul *et al.,* 2011) and was carried out in R as described above for the functional genes. Shannon diversity was calculated using R's package phyloseq. Data were subsampled to equal depth (42 270 sequences) before analysis. All sequences (*but, buk, 16S rRNA*) are available via SRA (PRJEB6978).

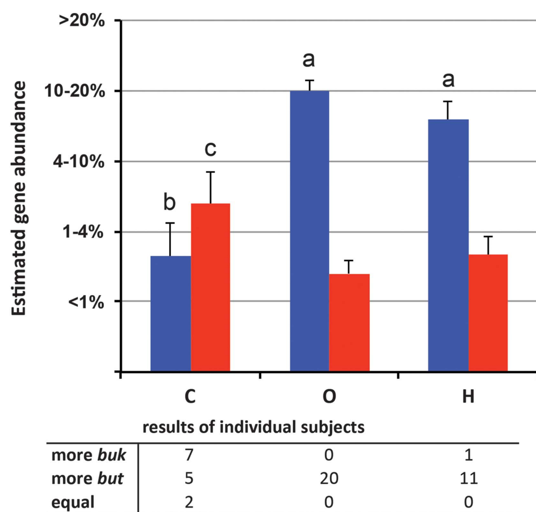
Results

The aim of the present study was to reveal the butyrate-producing communities in a broad set of hindgut-fermenting mammals, and members of the

related classes birds and reptiles, to investigate their association with diet and host phylogeny. A list of all animals including their diet and taxonomy is given in Table 1. Butyrate-producing communities were explored in depth using a functional gene-based pyrosequencing approach targeting the terminal genes of the main butyrate-synthesis pathways namely *but* and *buk* (Vital *et al.,* 2013). *16S rRNA* gene analysis served as a reference method.

Estimation of but and buk gene abundances in individual diet groups

Gene abundance estimations based on band intensities of amplified genes revealed abundant butyrate-producing communities in almost all fecal samples. A clear diet-specific pattern was observed, where estimated *but* gene abundances were significantly enriched in omni- and herbivores with an average closest to the category representing 10%–20% of the total community compared with an estimated average abundance positioned between categories of 1%–4% and <1% in carnivores (Figure 1). Estimated average percentages of the communities harboring *buk* trended to be increased in carnivores compared with omnivores ($P=0.051$), although not so compared with herbivores, $P=0.27$. Overall, *but* dominated the butyrate-producing community in omni- and herbivores ($P \ll 0.01$) where 100% and 92% showed a positive *but* to *buk* ratio, respectively, whereas *buk* was more abundant in many carnivorous animals



	C	O	H
more *buk*	7	0	1
more *but*	5	20	11
equal	2	0	0

Figure 1 Average gene abundance estimates for butyryl-CoA :acetate CoA-transferase (blue) and butyrate kinase (red) for individual diet groups (C: carnivores, O: omnivores, H: herbivores) are shown. Abundances were estimated by categorizing band intensities of PCR products into six distinct brightness groups (0–5) that were related to standard curves established with reference genomes. a: Differ significantly within diet groups $P \ll 0.01$, b: significantly reduced in carnivores ($P<0.01$), c: different to omnivores ($P=0.051$) and to herbivores ($P=0.27$). The s.e.m. is indicated. At the bottom the dominant gene for each subject of individual diet groups is shown.

(50%; Figure 1). However, a few carnivorous animals, especially the two arctic foxes and ferret, exhibited high *but* gene abundances. Noncarnivorous *Carnivora* followed the overall results of carnivores with significantly reduced estimated *but* gene abundances ($P<0.01$; data not shown) and dominance of *buk* in several subjects. Individual results for each sample are presented in Supplementary Figure S1. No significant estimated abundance differences were found for total butyrate-producing genes and individual *but* and *buk* genes, respectively, between vertebrate classes, whereas within the *Mammalia but* was significantly reduced in *Carnivora* ($P<0.01$) compared with primates and rodents (data not shown).

Many unknown but and buk gene sequences were detected

Diversity analysis based on complete-linkage-clustering of protein sequences applying a 5% cutoff revealed a large (unknown) gene diversity (Figure 2). For *but,* 5141 different clusters were detected. Analysis of representative sequences (rep. seq.) for each cluster demonstrated that the majority of *but*-linked clusters were not closely related to any known sequences from the FunGene database. For example, in carnivorous communities only 43.1% of rep. seq. showed ⩾90% identity to a reference, followed by omnivores (38.4%) and herbivores (21.3%; Figure 2a). Human-derived communities exhibit many more clusters closely related to known sequences—65.6% of rep. seq. showed ⩾90% identity to a reference. For the *buk* gene, 2924 clusters were detected (Figure 2b). Individual diet groups displayed a similar pattern as *but*; however, even less clusters had a close identity to a reference and many exhibited a rep. seq. that displayed <80% identity to database sequences. *Buk* clusters linked to humans followed the overall trend of other categories. However, the median cluster size was not constant but considerably larger for clusters with a rep. seq. exhibiting ⩾95% identity for both *but* and *buk* (Figure 2). Nevertheless, whereas analysis based on each gene sequence indeed displayed steeper curves, a large amount of obtained sequences are not closely related to a reference (Supplementary Figure S2).

Diet-specific clustering

Ordination analysis based on the combined butyrate-producing community (*but* and *buk* results were merged) revealed a diet-specific clustering ($P<0.01$ based on PERMANOVA analysis), where carnivores formed a separate group from omnivores and herbivores, which did not robustly separate from each other (Figure 3). No significant differences in diversity (Shannon) were obtained between diet groups (data not shown). Noncarnivorous *Carnivora* ($n=6$) clustered closer to carnivores than

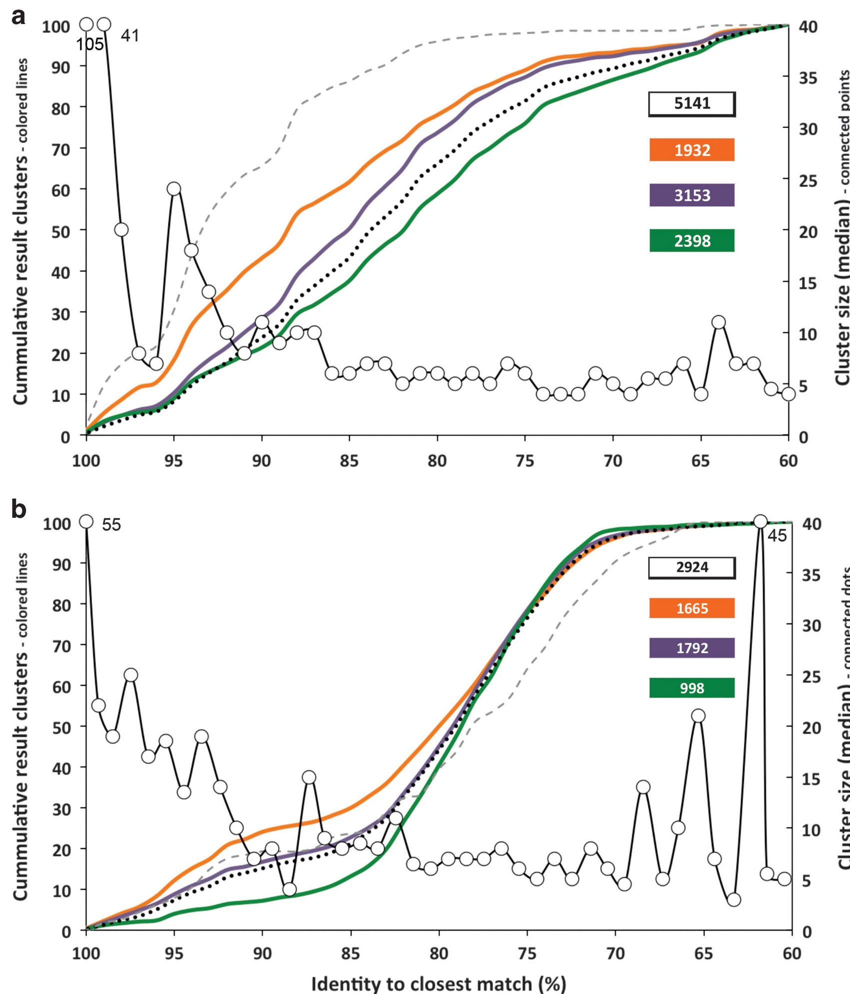


Figure 2 Diversity analysis of gene clusters (5% cutoff on protein level) of butyryl-CoA:acetate CoA-transferase (**a**) and butyrate kinase (**b**) is shown. Individual diet groups are indicated as orange (carnivores including noncarnivorous *Carnivora*), violet (omnivores), green (herbivores), gray-dashed line (human) and black-dotted line (all clusters). The data are displayed as a cumulative percentage of representative sequence identities to a reference gene in our database. Numbers of clusters per group are shown in boxes (individual diet groups associated with corresponding color, white: total clusters) and the median cluster sizes are indicated (numbers associated with certain points indicate the median cluster sizes that were above the axis-maximum of 40 sequences). Global singletons were not considered for analysis.

to members of their corresponding diet group. No significant grouping was found for individual vertebrate classes (data not shown); however, *Carnivora* formed a separate cluster from primates and rodents within the *Mammalia* (Supplementary Figure S3).

Closest match analysis also revealed distinct communities associated with different diets (Figure 4; individual results for each sample are presented in Supplementary Figure S4). All groups exhibited high percentages of *but* gene communities linked to several *Roseburia sp.* In carnivores, *but* genes associated with *Acidaminococcus sp. D21* were enriched, whereas genes related to 80_*Ruminococcacaea bacterium* D16 and 60_*Eubacterium hallii* L2-7 were specifically abundant in herbivores and omnivores. Simper analysis revealed several *Roseburia* sp., *Clostridium* sp. SS2/1 and 80_*E. rectale* (both down in herbivors), *E. limosum* (up in omnivores), 80_*Ruminococcacaea bacterium* D16 (down in carnivores), *Oscillibacter valericigenes* (up in herbivores) as well as *Clostridium symbiosum* WAL-14163 and 60_Megasphaera micronuciformis F0359 as the main contributors for dissimilarities between groups (Supplementary Figure S5). For *buk,* a clear separation of carnivores and herbivores was observed, where genes associated with *C. perfringens* and 80_*C. difficile* were significantly abundant in the former and the latter predominantly exhibited *buk* sequences related to 70/80_*Lachnospiracae bacterium* 3 1 57FAA CT1 and 70/80_*Coprococcus* sp. ART55/1, which were both detected as the most dissimilar taxa (next to *C. perfringens*) based on simper analysis. Omnivores were characterized by an 'in-between' pattern displaying abundant gene communities that were specific for the two other diets.

16S rRNA gene analysis

Overall, *16S rRNA* analysis grouped samples according to diet confirming results on *Mammalia* reported earlier by (Ley *et al.*, 2008; Supplementary Figure S6A). Similar to the functional gene results, noncarnivorous *Carnivora* grouped closer to *Carnivora* than to their individual dietary partners (result not clear for the two dog samples). A reduced bacterial diversity (Shannon) for carnivores compared with other two diet groups was observed (Supplementary Figure S6B). Results on the phylum level are presented in Supplementary Figure S7. Specific analysis for butyrate-producing taxa on the genus level showed a similar overall pattern as the functional gene-targeted approach. Genera linked to *buk* were enriched in carnivores, whereas *but*-linked taxa were much more abundant in omnivores and herbivores (a list of butyrate-producing genera found in the data is presented in Supplementary Table S1). *But* gene communities primarily consisted of *Lachnospiraceae* (in particular *Clostridium*

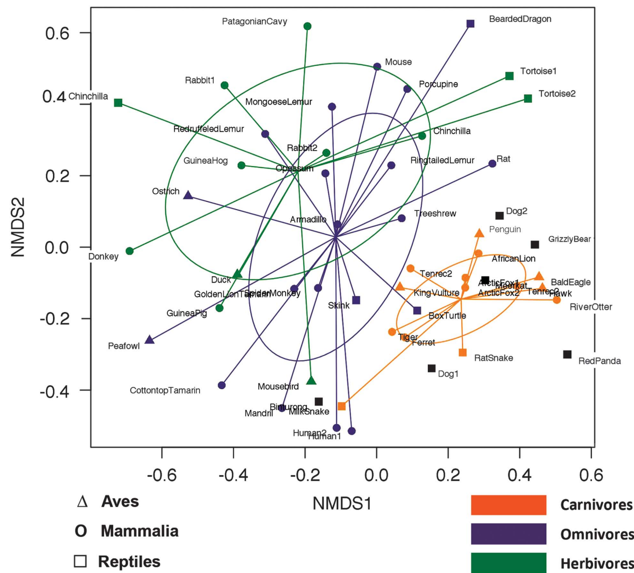


Figure 3 Nonmetric multidimensional scaling (NMDS) of combined gene data (butyryl-CoA:acetate CoA-transferase and butyrate kinase) based on multilinkage clustering (5% cutoff on the protein level) is shown (stress = 0.27). Individual diet groups are indicated as orange (carnivores), violet (omnivores) and green (herbivores). Distinct *Vertebrate* classes are specified with symbols. Noncarnivorous *Carnivora* are presented as black squares. Ellipses represent the s.d.'s of points. Diet was revealed as a significant factor ($P<0.01$ based on permutational ANOVA analysis).

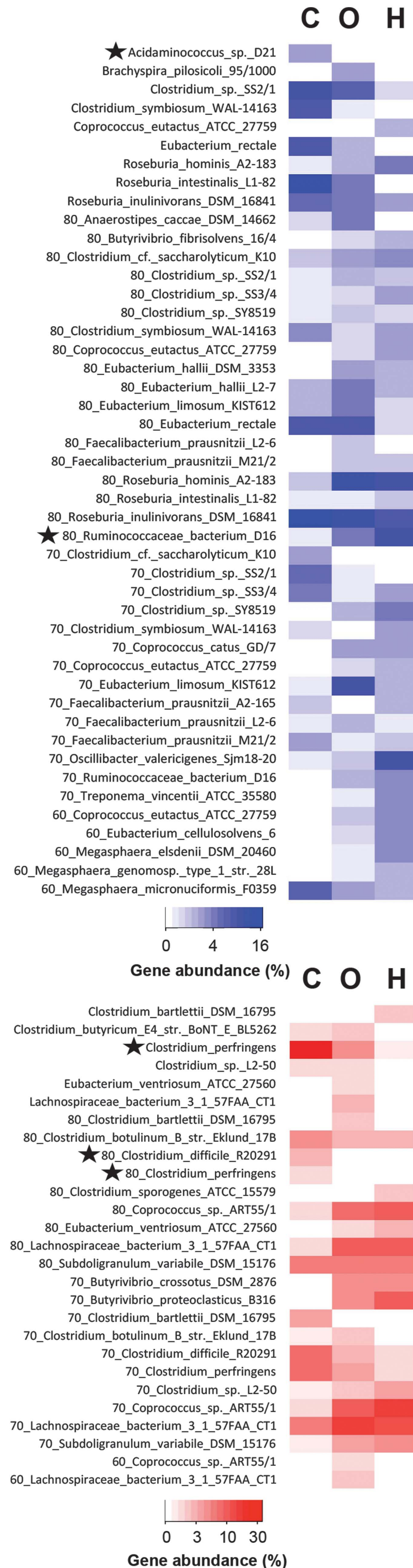


Figure 4 FrameBot closest match analysis of butyryl-CoA:acetate CoA-transferase (blue) and butyrate kinase (red) for individual diet groups (C: carnivores and noncarnivorous *Carnivora*, O: omnivores, H: herbivores) is shown. Closest match results were separated into distinct categories based on their percent identity to a reference gene (bins containing 10% identity ranges were created, where the number preceding the reference name specifies the lowest identity in that group; for example, '70_reference name' is the combined percentage of all sequences that show 70%–79% identity to that reference; no number indicates categories from 90% to 100% identity). Only taxa accounting for more than one percent of the entire gene community are shown. A black star indicates taxa significantly difference ($P<0.05$) between diet groups.

XIVa sp., *Lachnospiracea incertae sedis* sp. and *Roseburia* sp.), *Ruminococcaceae* (mainly *Faecalibacterium* sp.) and *Spirochaetaceae (*only *Treponema* sp.), whereas *Clostridium sensu stricto* sp. (*Clostridiaceae*) and *Clostridium* XI sp. (*Peptostreptococcaceae*) were the main *buk*-associated taxa. A few abundant genera contain members exhibiting either *but* or *buk,* such as *Clostridium sensu stricto* sp. or *Coprococcus* sp., and the applied gene association can not be regarded as strict for those taxa. Furthermore, not always all members of a certain genus are butyrate producers; this is especially true for bacteria derived from *Clostridium* XIVa and *Lachnospiraceae_insertae_sedis.* Functional predictions shown in Figure 5 should, hence, be regarded as an estimate. Genera linked to alternative transferases (*Carnobacterium* sp. and *Erysipelotrichaceae incertae sedis* sp.) were detected in a few samples as well. Furthermore, all diet groups displayed several samples that contained taxa that are specifically known to produce butyrate via amino-acid fermentation namely *Fusobacterium* sp., which were primarily detected in several carnivores (and noncarnivorous *Carnivora*), as well as *Porphyromonadaceae* (mainly *Porphyromonas* sp. and *Odoribacter* sp.) and *Alistipes* sp. (*Rikenellaceae*), which were enriched in omnivores and herbivores (Figure 5).

Pearson correlation analysis between the entire *but*- and *buk*-associated communities and other genera showed a clear separation between the two groups (negative correlation of r^2: -0.42) and unique correlations with other taxa (Figure 6). Most obvious was a positive correlation of *but*-related communities with the abundant *Bacteroides* sp. (r^2: 0.73) and *Prevotella* sp. (r^2: 0.56), whereas *Escherichia* sp. was associated with *buk*-linked taxa (r^2: 0.59).

Discussion

We detected abundant butyrate-producing communities in most samples analyzed, demonstrating that they are omnipresent across *Mammalia* and adjunct vertebrate classes. The enormous functional redundancy (distinct microbial taxa that perform the same function) of the microbiota, which is adapted to individual host diets, enables bacteria to produce butyrate under various nutritional conditions. Owing to the volatile nature of butyrate and the different masses/densities between samples as well as differences in times of sample collection after

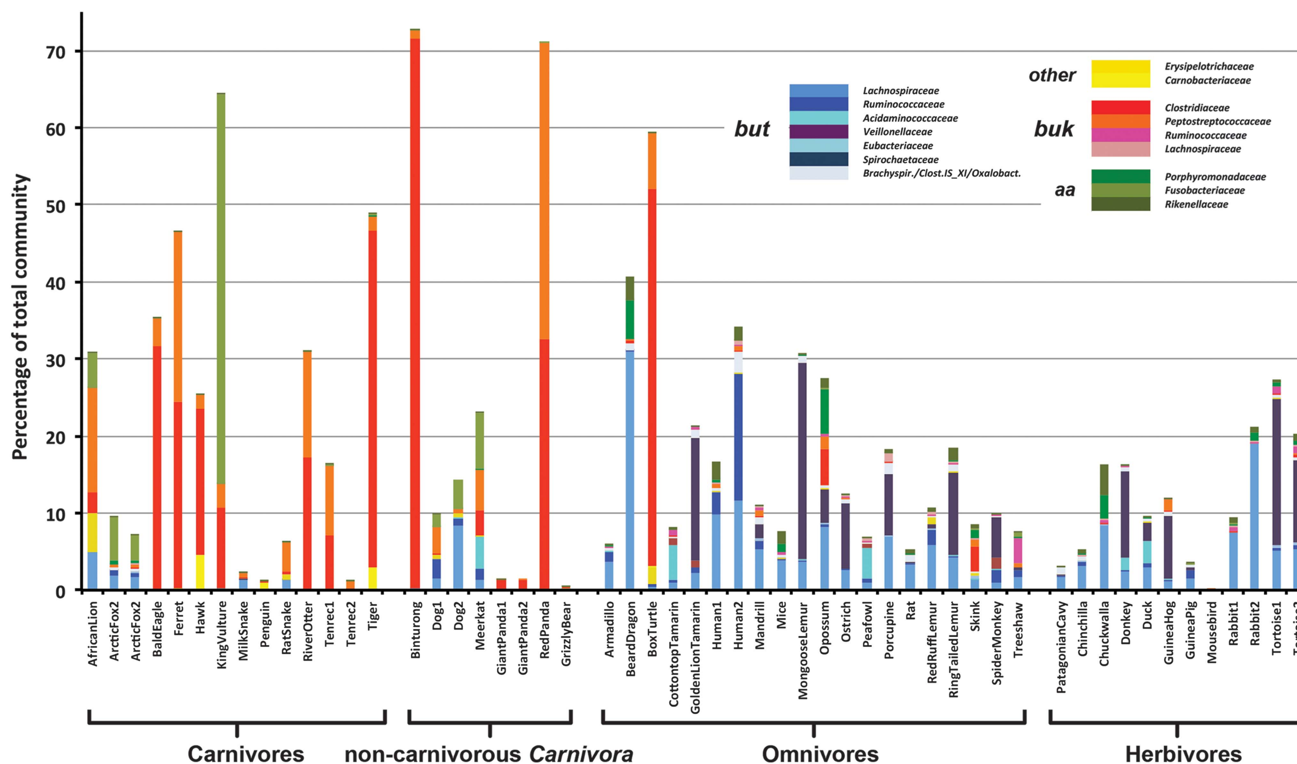


Figure 5 *16S rRNA* gene analysis for potential butyrate-producing taxa is shown as percentages of the total 16S *rRNA* gene sequences. Data were searched on a genus level and subsequently categorized into butyryl-CoA:acetate CoA-transferase (*but*: bluish colors) and butyrate kinase (*buk*; reddish colors)-linked taxa. The applied gene association should not be regarded as strict for those taxa as several genera contain both *but* and *buk* as well as non-butyrate-producing members (see main text). Thus, this type of analysis cannot substitute for a functional gene-targeted approach but is used here for global comparisons. Taxa associated with alternative transferases (other: yellowish colors) or amino-acid-fed butyrate-producing pathways (aa; greenish colors) are shown. Individual genera from the same family were combined (see Supplementary Table S1).

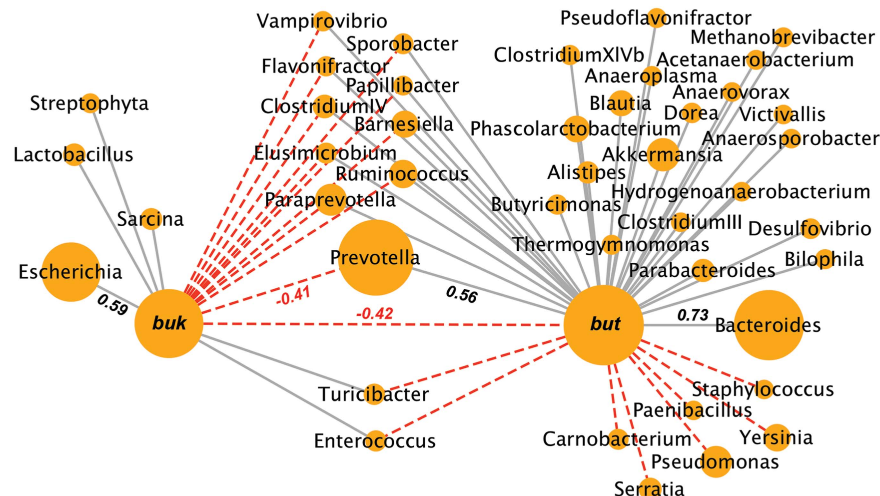


Figure 6 Pearson correlations of butyryl-CoA:acetate CoA-transferase communities (*but*) and butyrate kinase communities (*buk)* with other genera based on the *16S rRNA* results is presented. Genera linked to individual genes (*but* or *buk*) were combined before analysis. The type of correlation (gray line: positive, red-dashed line: negative) is indicated. The size of nodes is proportional to their overall abundance in our samples. Only significant correlations ($P < 0.01$) exhibiting an r^2 of > 0.4 were considered. r^2 values for most important correlations are shown.

defecation (1–24 h), accurate measurements of butyrate were not feasible. However, short-chain fatty acid measurements in fecal content of various vertebrates under controlled conditions found in literature support our results of a consistent butyrate-producing potential across members of this taxon. Stevens and Hume (1998) report high hindgut short-chain fatty acid concentrations in a series of comparative studies for carnivores, omnivores and herbivores with similar ratios between acetate-to-propionate-to-butyrate. Comparable concentrations were found in birds and reptiles (Stevens and Hume, 1998). Furthermore, *in vitro* incubation experiments of fecal matter showed similar butyrate-synthesis rates for species across dietary groups (Sunvold and Hussein, 1995). This strong conservation of butyrate synthesis across host taxa indicates that microbiota host interactions via butyrate are not specific to certain mammalian groups, but are, potentially, ubiquitous in vertebrates. Additional studies investigating several vertebrate taxa from distinct classes, specifically focusing on how butyrate affects host physiology, are needed in order to gain more insights into this apparent symbiosis.

Both methods, the functional gene approach as well as *16S rRNA* analysis, revealed similar overall results and complement each other well. However, specifically targeting *but* and *buk* allowed deeper insights into the butyrate-producing community at fine resolutions as certain genera include both producers and non-producers and some abundant genera such as *Clostridium sensu stricto* or *Coprococcus* exhibit members linked to *but* and others to *buk.* Furthermore, a few bacteria, which were frequently detected by the functional gene approach, in particular *Lachnospiraceae bacterium* 3_1_57FAA_CT1 and *Ruminococcaceae bacterium* D16, are not classified to the genus level. In total, a considerable percentage of sequences were not classifiable at the genus level (median: 20.4%, range: 0.51%–66.2%, data not shown). Those caveats of the *16S rRNA*-based analysis are most probably the reason why this method revealed much less *buk*-linked communities in both omni- and herbivores compared with the functional gene-based results.

The gene diversity analysis presented in Figure 2 (as well as Figure 4, Supplementary Figure S4) demonstrates a huge gap between isolated, sequenced organisms and actual *in vivo* communities in animal-derived samples. Most intestinal isolates and associated genome sequences in public databases are anthropocentric (many derived from the Human Microbiome Project (HMP, 2012)), leaving the microbiota of other hosts unknown. This was reflected in our data, where the majority of human-derived *but*-clusters, which is the predominant terminal butyrate-synthesis gene in this taxon (Louis *et al.,* 2004), display much higher identity to known references compared with other samples. However, even in human samples the majority of the less prevalent *buk* gene communities are not closely related to any reference. Our data suggest that additional sequencing efforts specifically targeting bacterial genomes from distinct host taxa are needed to close the current gap.

Communities differed considerably between diets where in particular carnivores displayed a unique pattern distinct from omnivores and herbivores. *Buk* gene communities linked to taxa that are associated with protein-rich environments and require several amino acids for growth, namely *C. difficile* and *C. perfringens* (Haslam *et al.,* 1986; Shimizu *et al.,* 2002), were prevalent in this group suggesting how butyrate production of the microbiota is adapted to a carnivorous diet. The low amount of *but* genes

in the majority of carnivores might stem from negative selection because of a lack of a consistent external acetate supply that is required for this gene to perform (Duncan *et al.,* 2004). Pearson correlations suggest that the strict anaerobic, abundant, acetate producers *Bacteroides* sp. and *Prevotella* sp. are strongly positively correlated to *but* gene communities supporting the proposed *but*-external acetate link (Figure 6). Although acetate production is also known for *Proteobacteria* that are abundantly found in carnivores (in particular *Escherichia* sp.), environmental conditions can change their physiologies towards consumption (Kleman and Strohl, 1994). This inconsistency renders functional co-evolution based on acetate production with other taxa unlikely. Thus, in this context the predominance of *buk* genes in carnivores makes sense. However, additional experiments specifically investigating the link between acetate concentration and *but* gene abundance as a possible co-evolutionary process are needed in order to verify our hypothesis. Interestingly, a study analyzing the short-chain fatty acid contents in Grizzly Bear feces showed high butyrate concentrations and only minimal concentrations of acetate indicating non-*but* butyrate synthesis in those individuals (Schwab *et al.,* 2009).

Although the acetyl-CoA pathway was abundant in most analyzed samples based on both methods, *16S rRNA* analysis additionally revealed certain genera that are known to exhibit alternative, protein-fed, butyrate-synthesis pathways (Barker *et al.,* 1982, Vital *et al.,* 2014; Figure 5). Interestingly, they were not more abundant in carnivores but are detected at similar levels across members of all diet groups. This is in accordance with metagenomic results presented earlier (Muegge *et al.,* 2011), where the increased abundance of amino-acid degradation pathways in carnivores did not result in enrichments of genes specifically associated with amino-acid-fed butyrate-synthesis pathways (based on EC numbers) and suggests that the distinct strategy in the terminal step of the acetyl-CoA pathway favoring either using *but* or *buk* is indeed the predominant adaptation process of the microbiota between animals of individual diet groups. However, those samples were not sequenced very deeply and investigations using new sequencing techniques could add additional information. In a recent study, human subjects who were exposed to diets composed of animal- or plant-derived products displayed functional communities that mirrored differences between herbivorous and carnivorous mammals (based on Muegge *et al.* (2011)) namely a distinct trade-off between carbohydrate and amino-acid degradation (David *et al*., 2013). From a butyrate-producing perspective, it is, however, unclear whether the human microbiota has the potential to adapt to those extreme diets in a similar way as found for different animals in this study. This is in particular in question for a strict carnivorous diet as butyrate production was significantly reduced in those human subjects (David *et al*., 2013). Our study revealed specific *buk*-containing *Clostridia* species as the key for butyrate synthesis in carnivorous animals and, although OTUs linked to *Clostridium* sp. were indeed reported to increase in individuals consuming the animal-based products, it is unknown whether those taxa are linked to butyrate production and whether they particularly exhibit the *buk* gene. Furthermore, abundant butyrate-producing taxa in carnivorous animals such as *C. difficile* or *C. perfingens* are associated with disease in humans (Rood and Cole, 1991; Kelly and LaMont, 2008) and negative selection processes of the host might hinder their establishment.

Most noncarnivorous *Carnivora* exhibited communities that were closer to those of carnivores than to those of their dietary partners (Figure 3), which is analogous to the observed pattern of entire microbial fecal communities (Supplementary Figure S6A and Ley *et al.,* 2008). This illustrates that the governance of diet over the composition of butyrate-producing communities is not all-inclusive but that phylogenetic factors have a certain role as well. *Carnivora* are hindgut fermenters; however, they exhibit a distinct gut anatomy namely a so-called simple gut that might create additional selective environments for specific microbes explaining the non-diet-related similarities of their microbiota. Giant panda was the only animal that did not exhibit any butyrate-producing genes indicating that the extreme combination of a carnivorous gut anatomy with short retention times and a strict herbivorous diet (Dierenfeld *et al.,* 1982) does not allow the establishment of a butyrate-producing community. However, the related herbivorous red panda, which also belongs to the *Carnivora*, exhibited abundant *buk*-associated butyrate producers that are typical for that group. *Escherichia* sp. overwhelmingly dominated the microbial flora in our giant panda samples (data not shown). It was previously reported that captive animals display *Proteobacteria*-enriched communities at the expense of *Clostridia* (containing potential butyrate-producing bacteria), which prevail in wild individuals (Zhu *et al.,* 2011). This hints that specific unknown factors possibly associated with their captivity are biasing results and indicates that the observed absence of butyrate producers may not be normal for giant pandas.

This study presents the first assessment on butyrate-producing communities over a broad range of vertebrates ($n = 54$) and adds substantial new information on the diversity of this important microbial function as well as its adaptation strategies to distinct diets and hosts. It demonstrates that functional properties can be conserved in bacterial communities, despite substantial differences in its structure illustrating the need of functional-based investigations to reveal specific host–microbiota interactions.

Conflict of Interest

The authors declare no conflict of interest.

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