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Authors: Menke, Sebastian, Heurich, Marco, Henrich, Maik, Wilhelm, Kerstin, and Sommer, Simone

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Impact of winter enclosures on the gut bacterial microbiota of red deer in the Bavarian Forest National Park

Sebastian Menke, Marco Heurich, Maik Henrich, Kerstin Wilhelm and Simone Sommer

S. Menke, K. Wilhelm and S. Sommer (simone.sommer@uni-ulm.de), Inst. of Ecology and Conservation Genomics, Univ. of Ulm, Albert-Einstein Allee 11, DE-89081 Ulm, Germany. – M. Heurich and M. Henrich, Dept of Conservation and Research, Bavarian Forest National Park, Zoology, Grafenau, Germany.

High numbers of red deer *Cervus elaphus* pose a challenge for natural forests because of their high browsing intensities, especially during winter months. To mitigate this human–wildlife conflict, conservation management in central Europe involves luring red deer into fenced winter-feeding sites. The supplementary food provided in these so-called winter enclosures strongly differs from the natural diet of red deer. Dietary shifts, however, can lead to an imbalance of the gut microbiota, which could promote bacterial pathogens. Moreover, increased inter-individual contact in winter enclosures enhances the exchange of symbiotic but also pathogenic bacteria. In this study, we used high-throughput sequencing of the 16S rRNA gene in fecal samples of red deer inhabiting the Bavarian Forest National Park to investigate differences in the gut bacterial microbiota between individuals in winter enclosures and individuals that ranged freely in the forests in winter. We also investigated the occurrence of potential zoonotic bacterial pathogens in both study groups. Our results revealed that proportions of bacterial taxa, alpha- and beta-diversities, and relative abundances of amplicon sequence variants in the gut bacterial microbiota of the two groups differed. These differences were attributed to the enrichment of bacterial taxa involved in the digestion of the supplementary food and to different natural diets consumed before entering the winter enclosures. We detected sequences with high similarities to known red deer pathogens in both study groups, but their relative abundances were low, which suggests that the population of red deer of the Bavarian Forest National Park is healthy.

Keywords: 16S rRNA gene, supplementary food, wildlife management

Large herbivores are drivers of important processes that can shape the composition and structure of forest ecosystems (Gill and Beardall 2001, Rooney and Waller 2003, Tremblay et al. 2007). Their consumption of vegetation affects the growth and survival of many herb, shrub, and tree species and modifies patterns of the relative abundance and competition of these plants (Côté et al. 2004). Moreover, seed dispersal, urination, defecation, and the resource pulse after death of an herbivore can create heterogeneity at various spatial and temporal scales (Bardgett and Wardle 2003, Selva Fernández 2004, Pellerin et al. 2016). Although these diverse processes caused by large herbivores are important for forest ecosystems, these activities can cause significant damage in managed forests. In particular, browsing and bark stripping are major obstacles to nature-oriented forestry (Hothorn and Müller 2010). Wildlife management in such set-ups is difficult because the well-being of the protected species, protection of natural processes, and limitation of forest damage in the surroundings of protected areas need to be considered (Schaller 2007, Gerner et al. 2012). At the same time, human intervention needs to be kept to a minimum (Günther and Heurich 2013).

In Europe, this conflict of interest has increased because populations of ungulates, especially red deer *Cervus elaphus*, have grown enormously in recent decades mainly because of hunting management, changes in agricultural practices and extermination of large carnivores (Apollonio et al. 2010). The impact of red deer on forests is especially high during winter months when the animals shift from a diverse diet to a diet consisting to a large extent of coniferous and deciduous trees (Krojerová-Prokešová et al. 2010). A common management tool in the Alps for the reduction of browsing pressure is the use of winter enclosures (Belotti et al. 2014, Heurich et al. 2015). In the Bavarian Forest National Park supplementary food is placed in the enclosures to attract red deer, starting in October. In November, the gates are closed; animals that arrive later are caught in a pre-enclosure before they are allowed to enter the main enclosure. The red deer

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remain in captivity until the beginning of the vegetation period at the end of April or in May, depending on weather conditions. This management measure strongly reduces bark stripping and browsing during winter (Heurich et al. 2015).

Research into the impact of such winter enclosures and supplementary feeding has revealed both positive and negative results (Putman and Staines 2004). Positive aspects include increased winter survival (Peterson and Messmer 2007), less forest damage (Gundersen et al. 2004), more efficient population control (Heurich et al. 2011), and better trophy quality (Putman and Staines 2004). Negative aspects, in addition to the high costs related to the maintenance of winter enclosures and food provision (Putman and Staines 2004), include increased browsing activity close to the winter enclosures (Putman and Staines 2004, Möst et al. 2015), altered spatial behavior (Sahlsten et al. 2010, Ossi et al. 2017), increased stress (Li et al. 2007), and risk of disease transmission attributable to the high animal densities at shared feeding sites (Santín-Durán et al. 2004, Cotterill et al. 2018). In addition to these acknowledged impacts, this management tool might also cause alterations in the symbiotic gut microbiota of these large herbivores. Ruminants have evolved a special digestive compartment, the so-called forestomach, in which symbiotic microbes produce essential enzymes that are needed to degrade plant material before it enters the true stomach (Soest 1994). It has been observed that animals coming to winter enclosures develop a reliance on food supplements, change their feeding strategies (Felton et al. 2017), and hardly feed on natural food sources (Putman and Staines 2004). Therefore, supplementary feeding in winter enclosures could cause significant shifts in the gut microbial community of herbivores that even exceed the normal range of individual variation within a population, with unknown consequences for their health.

Recent studies of the bacterial microbiota of humans and wildlife have demonstrated that a balanced host–bacteria relationship is important for host health (Carding et al. 2015, Jiménez and Sommer 2016, Thomason et al. 2017, Wasimuddin et al. 2018). In homeostasis, bacterial communities can, among many other beneficial functions, facilitate the uptake of vital nutrients from the diet, counteract infections (Jiménez and Sommer 2016, Walke and Belden 2016, Wasimuddin et al. 2017), degrade/decompose toxic plant secondary compounds (Kohl et al. 2014), and even signal the membership of a host to a certain social group via bacterial-derived odors (Leclaire et al. 2014).

In a state of dysbiosis, however, the gut microbiota can cause an increase in potential pathogens, which can have adverse effects on host health (McKenna et al. 2008, Kinross et al. 2011, Amato et al. 2013, Althani et al. 2016). The susceptibility of red deer to pathogenic bacteria could also be affected by the elevated intra-specific contact at shared feeding sites (Miller et al. 2003). For example, reintroduced red deer in Slovakia, Hungary and Poland carry zoonotic pathogenic taxa (Gnat et al. 2015). Thus, the close contact to humans at winter enclosures might also increase the likelihood of zoonosis transmission. In addition, in contrast to the vast amount of research on dietary effects on the human gut microbiota and dysbiosis-associated diseases (David et al. 2014, Althani et al. 2016), surprisingly little work has been carried out on the impact of supplementary feeding on the gut bacterial community of our most abundant large herbivores (but see: Li et al. 2017a, Gnat et al. 2018).

In this study, we used a 16S rRNA gene high-throughput sequencing approach 1) to describe the gut bacterial microbiota of red deer in the Bavarian Forest National Park, 2) to test whether the gut bacterial microbiota of red deer that spend the winter in enclosures differs from that of those that freely range in the forest, and 3) to study whether potential zoonotic pathogenic bacterial taxa occur in Bavarian red deer. An understanding of the effects of such regimes on the gut bacterial microbiota might add new but so far neglected aspects to the discussion of whether winter enclosures and supplementary feeding are good tools of red deer and forest health management.

Material and methods

Fecal sampling

The Bavarian Forest National Park (Bavaria, Germany) currently maintains four winter enclosures, namely Ahornschachten, Buchenau, Neuhüttenwiese and Riedlhäng (Fig. 1), which harbor approximately 0.86 to 4.2 red deer ha^{-1} during the winter months (Table 1). The supplementary food provided to red deer in these winter enclosures consists mainly of silage fodder with added apple pomace and sugar beets.

Fresh fecal samples from red deer in the four winter enclosures were collected randomly from 9 to 16 March 2017 (Ahornschachten, n = 24; Buchenau, n = 25; Neuhüttenwiese, n= 25; and Riedlhäng, n =25). In addition, fecal samples were collected during the same period from free-ranging deer at two locations outside of the winter enclosures, namely Reschbachtal (n = 23) and Hochberg (n = 14) (Fig. 1); these sites were chosen based on information obtained from experienced game wardens.

To avoid environmental bacterial contamination, fresh samples were opened in the field with sterile equipment, and the inner part of each fecal dropping was retrieved and transferred to a cryo tube containing RNAlater to preserve the samples. Samples were then stored at −20°C at the laboratory facilities of the Bavarian Forest National Park until they were sent to the Univ. of Ulm for DNA isolation, library preparation, high-throughput sequencing, and bioinformatic analyses.

DNA extraction, library preparation and sequencing

Prior to DNA extraction, samples were pre-treated to remove excess RNAlater and to remove non-bacterial matter. Fingernail-sized portions of fecal matter were transferred to 1.5 ml Eppendorf tubes using a spatula. Ice-cold phosphate-buffered saline (PBS; 400 µl) was added to each tube, and the tubes were vortexed for 10–15 s. The samples were lightly centrifuged at 700 rpm for 2 min. The supernatants were transferred to new 1.5 ml Eppendorf tubes, taking precautions to minimize the transfer of pelleted plant material, and centrifuged again, but this time at 14 000 rpm for 10 min. The supernatants were

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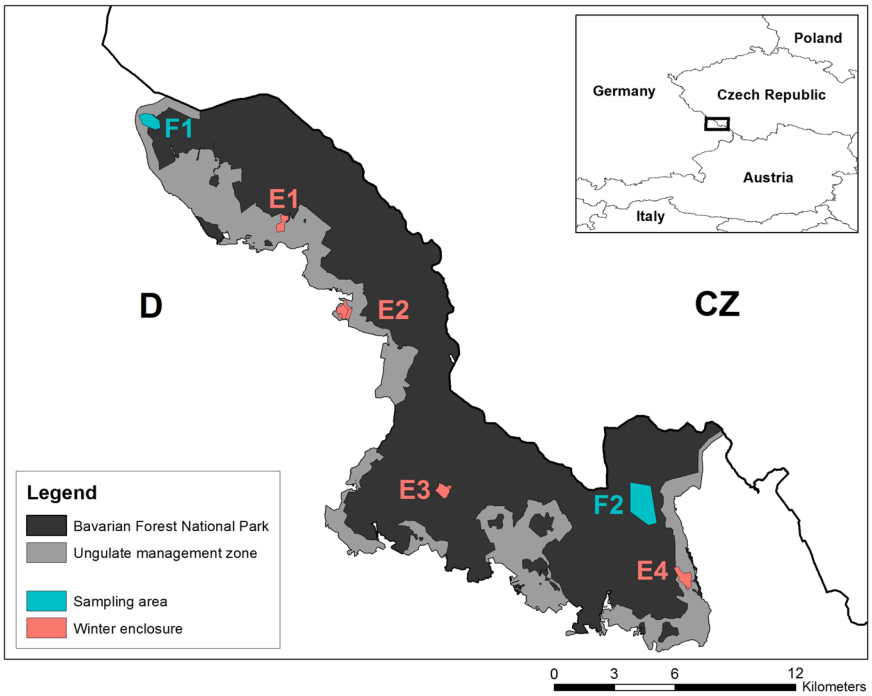


Figure 1. Map of the Bavarian Forest National Park showing the sampling sites of red deer in enclosures (E1: Riedlhäng, E2: Neuhüttenwiese, E3: Buchenau, E4: Ahornschachten) and sampling sites of free-ranging red deer (F1: Reschbachtal, F2: Hochberg).

discarded, and the remaining pellets were used as the starting material for the NucleoSpinFood kit (Macherey-Nagel, Düren, Germany), following the user manual from July 2014/Rev.11. An additional bead-beating step (ceramic beads; beating twice for 20 s) to mechanically lyse bacterial cells was incorporated between the chemical lysis of sample cells with the provided CF Buffer and the DNA-binding step. After beating, the samples were centrifuged at 11 000 rpm for 2 min, and the clear supernatants were transferred to 2 ml Eppendorf tubes. The DNA concentration in each tube was normalized to 3 ng μl^{-1} based on measurements with a TECAN infinite F200PRO fluorescence plate reader. The DNA library of each sample was prepared as recommended by Illumina (Miseq Reagent Kit v2 Denature and Dilute Libraries Guide, no. 15039740 v05); 7.5 pm DNA was loaded into a MiSeq flowcell with a 10% PhiX spike. Paired-end sequencing was performed over 2 × 251 cycles.

Bioinformatic analyses and statistics

Sequencing reads were pre-processed using qiime2 (ver. 2017.10) (Caporaso et al. 2010, <https://qiime2.org>) and its plugins. Specifically, we used the 'demux' plugin (<https://github.com/qiime2/q2-demux>) to import the demultiplexed paired-end sequencing reads and to create the 'artifact' file (i.e. qiime2 data format required for subsequent analyses). We applied the 'dada2' plugin (Callahan et al. 2016) using the default parameter settings for quality filtering and chimera filtering to trim primers (--p-trim-left-f 23, --p-trim-left-r 20), to truncate forward and reverse reads (--p-trunc-len-f 200, --p-trunc-len-r 200), and to collapse reads into representative sequences, the so-called amplicon sequence variants (ASVs). We assigned taxonomy to these ASVs using the Greengenes database (ver. 13_8) and the 'feature-classifier' plugin (<https://github.com/qiime2/q2-feature-classifier>) with the 'fit-classifier-sklearn' method and produced taxa summary bar plots (<https://github.com/qiime2/q2-taxa>) according to sample groupings.

For diversity analyses based on bacterial phylogeny, we produced a mid-point-rooted bacterial phylogenetic tree by aligning ASVs using MAFFT (Katoh and Standley 2013) and removing non-informative positions in the alignment with the 'mask' command (<https://github.com/qiime2/q2-alignment>); and a phylogenetic tree was constructed with FastTree 2 (Price et al. 2010). The 'diversity' plugin (<https://github.com/qiime2/q2-diversity>) was used to calculate alpha-diversity (phylogenetic diversity; Faith 1992) based on 13 900 reads per red deer gut bacterial microbiota.

Table 1. Number of red deer that spent winter 2017 in the four winter enclosures of the Bavarian Forest National Park.

		Males				Females			
		Age				Age			
Winter enclosure	Total red deer	n ≥ 3 years	3–10 years	>10 years	Total males	>2 years	1–2 years	> 1 year	Total females
Ahornschachten	25	5	1		6	9	4	6	19
Buchenau	52	10	9	1	20	10	8	14	32
Neuhüttenwiese	137	23	25	12	60	44	15	18	77
Riedlhäng	68	16	9	3	28	21	9	10	40

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For further analyses and creation of figures in R (<www.r-project.org>), we exported the non-rarefied 'feature-table', bacterial phylogenetic tree, representative sequences, and bacterial taxonomy from qiime2 'artifacts'. Using the R-package 'phyloseq' (McMurdie and Holmes 2013), we imported the exported files (<http://biom-format.org>; McDonald et al. 2012) and added metadata information to our class object. We also exported values of phylogenetic diversity to R and tested for significant differences in phylogenetic diversity between sites and overwintering areas using a Kruskal–Wallis rank sum test. Within 'phyloseq', we calculated a beta-diversity matrix (Bray–Curtis; Somerfield 2008) based on 13 900 reads per gut bacterial microbiota and tested whether overwintering areas (enclosure versus forest) and sampling sites differed significantly using a PERMANOVA approach with the 'adonis' function of the R-package 'vegan' (Dixon 2003). Furthermore, we tested for within and between differences of beta-diversity distances using the 'anosim' function of the 'vegan' package in R (Dixon 2003).

In addition, we applied a DESeq2 analysis to identify bacterial ASVs whose relative abundances significantly differed between the gut microbiota of red deer in enclosures and that of free-ranging red deer (McMurdie and Holmes 2013, Love et al. 2014). Finally, we checked for potentially pathogenic bacteria in the red deer gut microbiota and specifically searched for bacterial taxa known to contain pathogens that had been detected in red deer from Slovakia, Hungary and Poland (Gnat et al. 2015) using BLAST searches (Johnson et al. 2008) of the respective ASVs against the NCBI nucleotide database.

Data deposition

Sequencing data is available in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.7r22vb1> (Menke et al. 2018).

Results

Sequencing of the bacterial 16S rRNA gene in fecal samples of 99 red deer in enclosures and 37 free-ranging red deer resulted in 10 053 298 reads. After preprocessing, the remaining 6 988 136 reads (min: 13 929; median: 51 607; mean: 51 383; max: 88 217) were collapsed into 11 105 ASVs (mean: 629.28 reads per ASV).

The gut bacterial microbiota of the red deer of both groups consisted mainly of the bacterial phyla (average proportion across all individuals >1%) Firmicutes (71.50%), Bacteroidetes (15.55%), Proteobacteria (4.90%), Actinobacteria (3.68%), and Cyanobacteria (1.21%) (Fig. 2). The gut bacterial microbiota of red deer in enclosures contained on average higher proportions of Firmicutes ($W = 3039$, $p < 0.001$) and Actinobacteria ($W = 1314$, $p = 0.011$), but particularly lower proportions of Proteobacteria ($W = 1141$, $p < 0.001$) than that of free-ranging red deer (Table 2). A summary of the best taxonomic assignments of the bacterial phyla in the feces of all individuals is presented as a table in the supplementary information (Supplementary material Appendix 1 Table A1).

Alpha-diversity, expressed as phylogenetic diversity (Faith and Baker 2007), was higher in the gut bacterial microbiota of red deer in enclosures than in that of free-ranging red deer (Kruskal–Wallis: $\chi^2 = 6.96$, $p = 0.008$, Fig. 3A). Phylogenetic diversity of all red deer ranged from 19.69 to 64.12 and was highest in the gut bacterial microbiota of red deer in the winter enclosures Ahornschachten (E) and Riedlhäng (E), intermediate in Reschbachtal (F) and Buchenau (E), and lowest in Neuhüttenwiese (E) and Hochberg (F) (Fig. 3B).

Red deer in enclosures and free-ranging red deer also differed in beta-diversity. An NMDS plot based on the Bray–Curtis distance matrix revealed clear separation of the two groups of red deer (PERMANOVA: $F = 14.42$, $p = 0.001$; Fig. 4A) and clustering according to sampling sites (PERMANOVA: $F = 16.68$, $p = 0.001$; Fig. 4B). The distances between the gut bacterial microbiota (within a 95% confidence interval) of red deer in Neuhüttenwiese (E) and Hochberg (F) were higher than those of all other sampling sites, and some gut bacterial microbiotas were far removed from the respective cluster centers (Fig. 4). In addition, distances between the gut bacterial microbiota of the two groups were higher than the distances within each group (ANOSIM: permutations = 999, $R = 0.623$, $p = 0.001$; Fig. 5A) and the distances were also higher within sampling

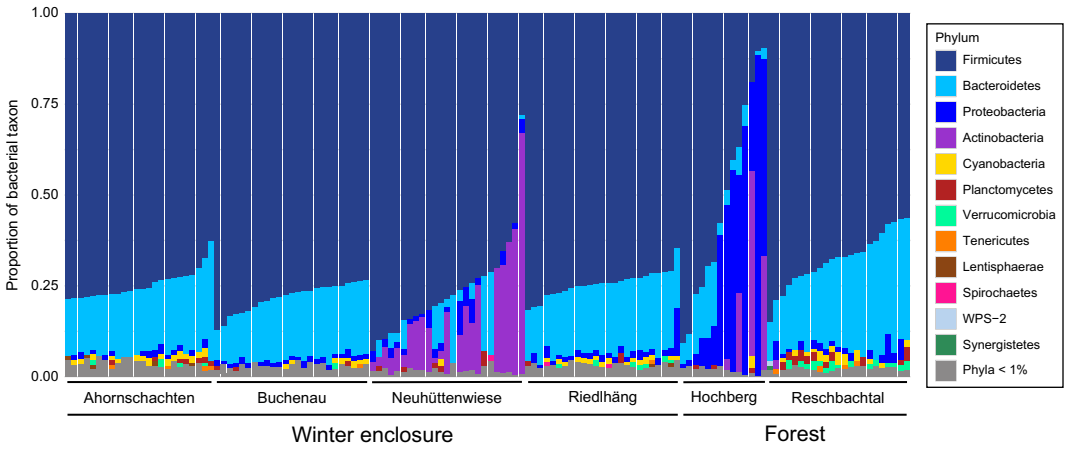


Figure 2. Proportions of bacterial phyla present in the gut microbiota of red deer from winter enclosures and forest sites of the Bavarian Forest National Park.

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Table 2. Average proportions of the main bacterial phyla (average proportion ≥ 0.5%) present in the gut bacterial microbiota of red deer in enclosures and free-ranging red deer in the Bavarian Forest National Park. The full list of phylum assignments is presented in the supplement (ST 1). Values in boldface are significant.

Phylum	Average proportion (%) of bacterial phyla of all red deer	Average proportion (%) of bacterial phyla of red deer in enclosures	Average proportion (%) of bacterial phyla of free-ranging red deer	Wilcoxon rank sum test (red deer in enclosures versus free-ranging red deer)
Firmicutes	68.11 ± 13.54	75.55 ± 7.45	60.66 ± 19.24	**W = 3039, p < 0.001**
Bacteroidetes	16.32 ± 8.42	14.65 ± 7.26	17.98 ± 10.70	W = 1432, p = 0.051
Proteobacteria	7.47 ± 12.45	1.83 ± 1.67	13.10 ± 21.89	**W = 1141, p < 0.001**
Actinobacteria	3.56 ± 9.96	3.83 ± 9.98	3.29 ± 10.02	**W = 1314, p = 0.011**
Cyanobacteria	1.34 ± 1.36	1.06 ± 0.59	1.61 ± 2.39	W = 1729, p = 0.618
Planctomycetes	0.89 ±0.72	0.71 ± 0.55	1.06 ± 1.01	W = 1628, p = 0.321
Verrucomicrobia	0.79 ±0.54	0.63 ± 0.41	0.94 ± 0.75	W = 1460, p = 0.070
Tenericutes	0.54 ± 0.32	0.55 ± 0.30	0.50 ± 0.36	W = 2181, p = 0.088

sites than the distances between the sampling sites (ANOSIM: permutations = 999, R = 0.418, p = 0.001; Fig. 5B).

DESeq2 analysis revealed that the relative abundance of several ASVs in the gut bacterial microbiota of red deer in enclosures significantly differed ($p < 0.001$) from that of free-ranging red deer (Fig. 6). In particular, the relative abundance of ASVs of the genera *Bacillus*, *Paenibacillus* and *Prevotella* was higher in the gut of red deer in enclosures, and the relative abundance of ASVs of the genera *Akkermansia*, *Bacteroides* and *Ruminococcus* were higher in the gut of free-ranging red deer.

Finally, we compared ASVs of bacterial genera that potentially contain zoonotic pathogenic bacterial species, as identified in Gnat et al. (2015), with the NCBI nucleotide database. The relative abundance of the genera *Escherichia*, *Yersinia*, *Enterococcus* and *Staphylococcus* was low (9%, 5%, 4% and 0.7%, respectively). BLAST results of ASVs of these genera revealed hits to known pathogens in red deer (Table 3).

Discussion

This is the first study to apply high-throughput sequencing to describe the gut bacterial microbiota of wild red deer and to compare the gut bacterial microbiota of individuals that stay in winter enclosures and feed on supplementary fodder with that of individuals that remain in the forest and feed only on natural resources. In general, the gut bacterial microbiota of red deer in the Bavarian Forest National Park was similar to that of other ruminants (Donnell et al. 2017) and also other Cervidae, such as elk *Cervus canadensis*, white-tailed deer *Odocoileus virginianus* (Gruninger et al. 2014), and white-lipped deer *Cervus albirostris* (Li et al. 2017b), at least at the bacterial phylum level, with high proportions of the phyla Firmicutes and Bacteroidetes (Fig. 2).

Particularly the phylum Firmicutes was present in higher proportions in red deer in enclosures than in free-ranging red deer. This is in concordance with a study in Poland in which red deer that were not fed with supplementary food during winter had a lower abundance of bacteria than red deer that were fed with supplementary food (Gnat et al. 2018). Recent studies on the microbiota of silage (Duniere et al. 2017, Peng et al. 2018) have revealed that certain bacterial taxa within the Firmicutes increase during ensiling when aerobic spoilage occurs (Driehuis et al. 2018). Thus, the Firmicutes probably increased in the gut microbiota of red deer in enclosures because they fed on silage. Interestingly, the relative abundances of phyla in the gut of red deer at two sites strongly differed from those of the other sites; the feces of red deer at the winter enclosure Neuhüttenwiese had higher proportions of Actinobacteria and lower proportions

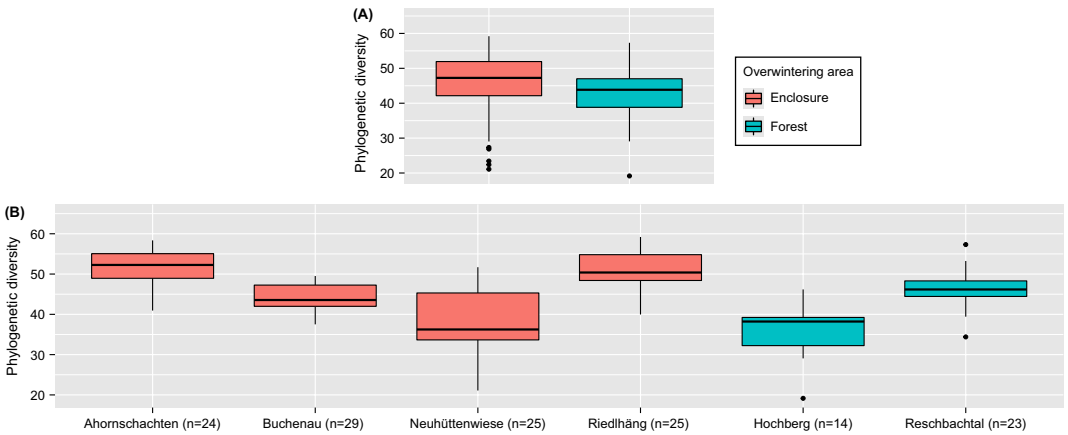


Figure 3. Phylogenetic diversity of the red deer gut bacterial microbiota. (A) Phylogenetic diversity of the gut bacterial microbiota was higher in individuals kept in enclosures than in red deer free-ranging in the forest (Kruskal–Wallis: $\chi^2 = 6.96$, $p = 0.008$). (B) The phylogenetic diversity between all pairs of sites significantly differed (Kruskal–Wallis test, all $p < 0.05$) except for that of Ahornschachten versus Riedlhäng.

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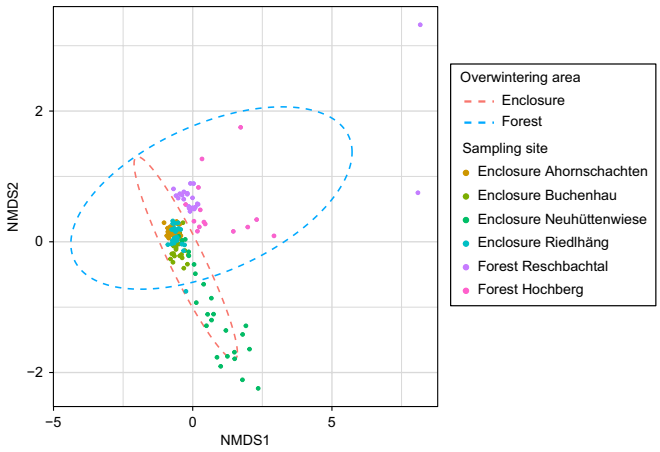


Figure 4. NMDS plot based on the Bray–Curtis distance matrix of the gut bacterial microbiota of red deer in enclosures. Significant differences in beta-diversity were found between sampling sites (PERMANOVA: F = 16.68, p = 0.001) and overwintering area (PERMANOVA: F = 14.42, p = 0.001).

of Bacteroidetes, and the feces of free-ranging red deer at Hochberg had higher proportions of Proteobacteria. Members of Actinobacteria play a central role in cellulose and lignin degradation (Le Roes-Hill et al. 2011, Zhu et al. 2011, Hanshew et al. 2015, Lewin et al. 2016), which is one of the most important functions provided by bacteria to large herbivores (Fang et al. 2012). Hunters in the Hochberg area attract red deer with apple pomace, which in combination with natural food sources might have created a distinct gut bacterial microbiota profile.

We found that both sampling site and overwintering area strongly affect alpha- and beta-diversities and that the overwintering area strongly affects the bacteria present in the red deer guts at the ASV level. Alpha-diversity differed between sampling sites and was higher in the gut of red deer in enclosures than in that of free-ranging red deer (Fig. 3), the gut bacterial microbiota of the two groups of red deer separated accordingly (Fig. 4), and the bacterial ASVs that strongly drove the observed differences between the two groups belonged to the genera *Bacillus*, CF231, *Paenibacillus* and *Prevotella* (more abundant in the gut of red deer in enclosures), and *Akkermansia*, *Bacteroides* and *Ruminococcus* (more abundant in the gut of free-ranging red deer).

Several interacting factors might be responsible for these observations. In a study of Limousin and Jinan crossbred steers, silage rich in nitrate led to an increase in the relative abundance of the bacterial taxon CF231 in the gut (Zhao et al. 2015). We did not check the nitrate level of the supplementary food in our study, but if nitrate levels were high because of incomplete reduction during ensiling or if they were higher than in natural food sources, this might have caused the observed increase of this genus in the gut of red deer in enclosures. As has been shown in studies on silage production, members of the phylum Firmicutes, especially

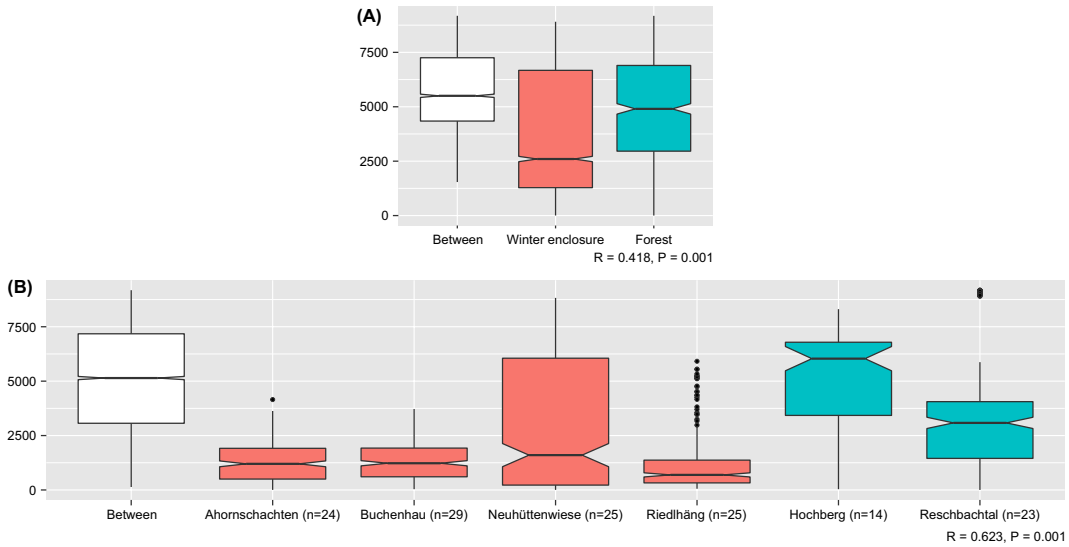


Figure 5. Bray–Curtis distances of the gut bacterial microbiota of red deer. (A) Bray–Curtis distances for gut bacterial microbiota of red deer in winter enclosures and free-ranging red deer at forest sites. (B) Bray–Curtis distances of gut bacterial microbiota of red deer within and between overwintering areas.

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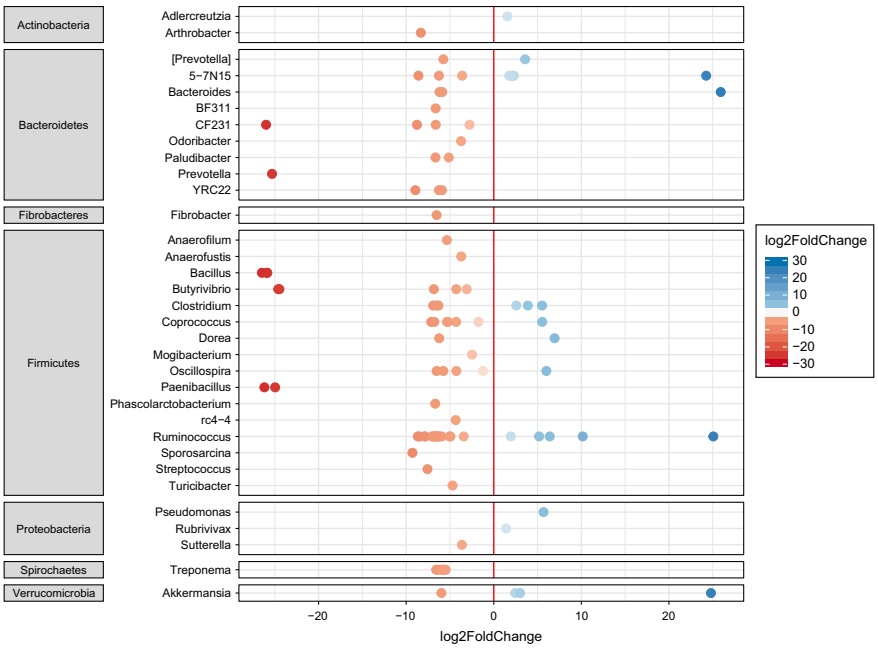


Figure 6. DESeq2 analysis of bacterial ASVs of the gut of free-ranging red deer and red deer in enclosures. Negative log2FoldChange values (red dots) represent ASVs that occurred at higher relative abundances in the gut of red deer in enclosures than in that of free-ranging red deer. Positive values (blue dots) represent ASVs that occurred at higher relative abundances in the gut of free-ranging red deer than in that of red deer in enclosures.

spores of *Bacillus* (Duniere et al. 2017) and *Paenibacillus* (Tohno et al. 2016), can increase in silage production during aerobic spoilage (Borreani et al. 2013). As red deer in enclosures fed almost exclusively on supplementary food, they probably ingested large amounts of spores of these bacteria, which we then detected in red deer feces. Spores of toxin-producing species of these genera present a huge problem in dairy milk production because of their ability to survive pasteurization and subsequent germination in the product (Gopal et al. 2015). However, in animals, spores usually only pass through the digestive system of the host and, to the best of our knowledge, do not affect host health (Driehuis and Elferink 2000).

Moreover, the proportion of *Ruminococcus* was higher in free-ranging red deer than in red deer in enclosures. Species of the genus *Ruminococcus* are important for the degradation and fermentation of dietary polysaccharides in ruminants (Julliand et al. 1999, La Reau et al. 2016) and require fermentable carbohydrates for growth (Rainey 2009). Fermentation during silage production might have led to a food source that is lower in fermentable carbohydrates compared to fresh food sources. The higher relative abundance of *Akkermansia* in the gut of free-ranging red deer is in contrast to results of a study on musk deer *Moschus berezovskii* in which the gut of captive individuals had a higher relative abundance of this bacterial genus than that of free-ranging deer (Li et al. 2017a). Nevertheless, the differences in the gut bacterial microbiota of red deer between the overwintering areas in our study were less pronounced than the differences identified in studies that compared wild and captive forest musk deer (Li et al. 2017a) and wild and captive sika deer *Cervus nippon hortulorum* (Guan et al. 2017). Thus, the difference in the gut bacterial microbiota among sampling sites is driven by a combination of local

Table 3. NCBI BLAST results (only the first result is shown) of ASVs of bacterial genera that contain pathogenic bacterial species (Gnat et al. 2015) in the guts of red deer.

Genus and ASVs	Number of individuals with taxon present in red deer in enclosures (n = 99) versus free-ranging red deer (n = 37)	BLAST hit	E-value	Ident	Accession
Escherichia					
ASV: d46e2205f0c6ecf67b51f83d111c509c	11 versus 2	*Escherichia coli*	3e-128	100%	MH588300.1
Yersinia					
ASV: 3481fa43fe5fba6aecdc7f9aae6ed9c0	6 versus 2	*Yersinia enterocolitica*	3e-128	100%	MH245052.1
Enterococcus					
ASV: 556864a5da3a811b67be9fc73488e926	2 versus 0	*Enterococcus thailandicus*	3e-128	100%	MH473433.1
ASV: 569653b1659271a290facfcedd0de061	1 versus 0	*Enterococcus gallinarum*	3e-128	100%	MH532500.1
ASV: a443e786f67086749ef29ce0ab494c9e	1 versus 0	*Enterococcus gilvum*	3e-128	100%	MH493694.1
ASV: e5e2076556acf09b18e99c982a375e75	1 versus 0	*Enterococcus asini*	3e-128	100%	KX688628.1
Staphylococcus					
ASV: c837e078de7918b20edaf786357d1eef	1 versus 0	*Staphylococcus epidermis*	6e-125	99%	KY623325.1

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factors, such as small-scale differences in food composition within enclosures (e.g. differences in silage quality) and in the forest area from which the animals were derived. Such local factors seem to lead to a variation between sampling sites without removing the features that lead to differences in the gut bacterial microbiota of red deer in enclosures and free-ranging red deer. The differences in the gut bacterial microbiota of red deer of forest sites might partly be attributable to the higher variation in local food sources from which free-ranging red deer can select (Krojerová-Prokešová et al. 2010). Data from GPS-collared red deer (unpublished data of the Bavarian Forest National Park) indicate that red deer usually return to the same winter enclosures. Therefore, one can reasonably assume that individuals that visit the same winter enclosures also forage in the same forest areas during the rest of the year. This would lead to higher similarities of their gut bacterial microbiota and further impact the distinctiveness of their gut bacterial microbiota between sampling sites because of a spatial differentiation in diet composition (Krojerová-Prokešová et al. 2010)

We identified four potentially pathogenic bacterial genera in red deer (*Escherichia*, *Yersini*, *Enterococus*, *Staphylococcus*), in contrast to the 12 taxa that were detected in red deer from Slovakia, Hungary and Poland (Gnat et al. 2015). The low numbers of reads and low relative abundance of the genera in our study suggests that red deer in the Bavarian Forest National Park are relatively healthy and rarely maintain bacterial zoonotic pathogens. *Escherichia coli* was more prevalent in the gut of red deer in enclosures than in the gut of free-ranging red deer, possibly because of interactions with humans in the enclosures. However, short fragments of the 16S rRNA gene, as used in this study, have their limitations in identifying bacterial zoonotic pathogens and other bacterial species with certainty. Thus, whether these *E. coli* strains are enterohemorragic seropathotypes (EHEC) needs further investigation.

In conclusion, our results revealed differences in the alpha- and beta-diversities of the gut bacterial microbiota of red deer in winter enclosures and free-ranging red deer. The strong difference in the relative abundance of members of the Firmicutes between the two groups of red deer is likely caused by the consumption of silage in enclosures. The guts of both groups of red deer carried known red deer pathogens, but their relative abundances were low, which suggests that red deer of the Bavarian Forest National Park represent a healthy population. Future studies should investigate the bacterial communities present in the supplementary food and apply metagenomics or transcriptomics on the red deer gut bacterial microbiota to reveal the impact of overwintering area at the level of bacterial functions. In addition, the pathogenicity of potentially pathogenic bacteria should be examined to identify their zoonotic potential.

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References

Althani, A. A. et al. 2016. Human microbiome and its association with health and diseases. – J. Cellular Physiol. 231: 1688–1694.
Amato, K. R. et al. 2013. Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. – ISME J. 7: 1344–1353.
Apollonio, M. et al. 2010. European ungulates and their management in the 21st century. – Cambridge Univ. Press.
Bardgett, R. D. and Wardle, D. A. 2003. Herbivore-mediated linkages between aboveground and belowground communities. – Ecology 84: 2258–2268.
Belotti, E. et al. 2014. Eurasian lynx hunting red deer: is there an influence of a winter enclosure system? – Eur. J. Wildl. Res. 60: 441–457.
Borreani, G. et al. 2013. Aerobic deterioration stimulates outgrowth of spore-forming *Paenibacillus* in corn silage stored under oxygen-barrier or polyethylene films. – J. Dairy Sci. 96: 5206–5216.
Callahan, B. J. et al. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. – Nat. Methods 13: 581–583.
Caporaso, J. G. et al. 2010. QIIME allows analysis of high-throughput community sequencing data. – Nat. Methods 7: 335–336.
Carding, S. et al. 2015. Dysbiosis of the gut microbiota in disease. – Microb. Ecol. Health Dis. 26: 26191.
Côté, S. D. et al. 2004. Ecological impacts of deer overabundance. – Annu. Rev. Ecol. Evol. Syst. 35: 113–147.
Cotterill, G. G. et al. 2018. Winter feeding of elk in the Greater Yellowstone Ecosystem and its effects on disease dynamics. – Phil. Trans. R. Soc. B 373: 20170093.
David, L. A. et al. 2014. Diet rapidly and reproducibly alters the human gut microbiome. – Nature 505: 559–563.
Dixon, P. 2003. VEGAN, a package of R functions for community ecology. – J. Veg. Sci. 14: 927–930.
Donnell, M. M. O. et al. 2017. Core fecal microbiota of domesticated herbivorous ruminant, hindgut fermenters and monogastric animals. – Microbiol. Open 6: e00509.
Driehuis, F. and Elferink, S. J. W. H. O. 2000. The impact of the quality of silage on animal health and food safety: a review. – Vet. Quart. 22: 212–216.
Driehuis, F. et al. 2018. Silage review: animal and human health risks from silage. – J. Dairy Sci. 101: 4093–4110.
Duniere, L. et al. 2017. Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. – BMC Microbiol. 17: 1–16.
Faith, D. P. 1992. Conservation evaluation and phylogenetic diversity. – Biol. Conserv. 61: 1–10.
Faith, D. P. and Baker, A. M. 2007. Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. – Evol. Bioinf. Online 2: 121–128.
Fang, W. et al. 2012. Evidence for lignin oxidation by the giant panda fecal microbiome. – PLoS One 7: e50312.
Felton, A. M. et al. 2017. Interactions between ungulates, forests, and supplementary feeding: the role of nutritional balancing in determining outcomes. – Mamm. Res. 62: 1–7.
Gerner, J. et al. 2012. How attitudes are shaped: controversies surrounding red deer management in a national park. – Human Dimensions Wildl. 17: 404–417.

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Gill, R. M. A. and Beardall, V. 2001. The impact of deer on woodlands: the effects of browsing and seed dispersal on vegetation structure and composition. – Forestry 74: 209–218.
Gnat, S. et al. 2015. Experimental studies of microbial populations and incidence of zoonotic pathogens in the faeces of red deer (*Cervus elaphus*). – Lett. Appl. Microbiol. 61: 446–452.
Gnat, S. et al. 2018. Effect of balanced supplementary feeding in winter on qualitative and quantitative changes in the population of microbes colonizing the rumen of red deer. – Medycyna Weterynaryjna 74: 6072–2018.
Gopal, N. et al. 2015. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. – Front. Microbiol. 6: 1418.
Gruninger, R. J. et al. 2014. Diversity of rumen bacteria in Canadian cervids. – PLoS One 9: e89682.
Guan, Y. et al. 2017. Comparison of the gut microbiota composition between wild and captive sika deer (*Cervus nippon hortulorum*) from feces by high-throughput sequencing. – AMB Express 7: 1–13.
Gundersen, H. et al. 2004. Supplemental feeding of migratory moose *Alces alces*: forest damage at two spatial scales. – Wildl. Biol. 10: 213–223.
Günther, S. and Heurich, M. 2013. Assessment of the naturalness of the red deer management in central European national parks. – Allgemeine Forst Jagdzeitung 184: 1–16.
Hanshew, A. S. et al. 2015. Characterization of Actinobacteria associated with three ant–plant mutualisms. – Microbiol. Ecol. 69: 192–203.
Heurich, M. et al. 2011. Management and conservation of large mammals in the Bavarian Forest National Park. – Silva Gabreta 17: 1–18.
Heurich, M. et al. 2015. Country, cover or protection: what shapes the distribution of red deer and roe deer in the Bohemian Forest ecosystem? – PLoS One 10: e0120960.
Hothorn, T. and Müller, J. 2010. Large-scale reduction of ungulate browsing by managed sport hunting. – For. Ecol. Manage. 260: 1416–1423.
Jiménez, R. R. and Sommer, S. 2016. The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. – Biodivers. Conserv. 26: 763–786.
Johnson, M. et al. 2008. NCBI BLAST: a better web interface. – Nucleic Acids Res. 36: W5–9.
Julliand, V. et al. 1999. Identification of *Ruminococcus flavefaciens*as the predominant cellulolytic bacterial species of the equine cecum. – Appl. Environ. Microbiol. 65: 3738–3741.
Katoh, K. and Standley, D. M. 2013. MAFFT multiple sequence alignment software ver. 7: improvements in performance and usability. – Mol. Biol. Evol. 30: 772–780.
Kinross, J. M. et al. 2011. Gut microbiome–host interactions in health and disease. – Genome Med. 3: 1–12.
Kohl, K. D. et al. 2014. Gut microbes of mammalian herbivores facilitate intake of plant toxins. – Ecol. Lett. 17: 1238–1246.
Krojerová-Prokešová, J. et al. 2010. Feeding patterns of red deer *Cervus elaphus* along an altitudinal gradient in the Bohemian Forest: effect of habitat and season. – Wildl. Biol. 16: 173–184.
La Reau, A. J. et al. 2016. Sequence-based analysis of the genus *Ruminococcus* resolves its phylogeny and reveals strong host association. – Microbiol. Genome 12: e000099.
Le Roes-Hill, M. et al. 2011. Actinobacteria isolated from termite guts as a source of novel oxidative enzymes. – Antonie van Leeuwenhoek 100: 589–605.
Leclaire, S. et al. 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. – Behav. Ecol. 25: 996–1004.
Lewin, G. R. et al. 2016. Evolution and ecology of Actinobacteria and their bioenergy applications. – Annu. Rev. Microbiol. 70: 235–254.
Li, C. et al. 2007. Influence of enclosure size and animal density on fecal cortisol concentration and aggression in Père David's deer stags. – Gen. Compar. Endocrinol. 151: 202–209.
Li, Y. et al. 2017a. Comparative analysis of the gut microbiota composition between captive and wild forest musk deer. – Front. Microbiol. 8: 1705.
Li, J. et al. 2017b. The gut bacterial community composition of wild *Cervus albirostris* (white-lipped deer) detected by the 16S ribosomal RNA gene sequencing. – Curr. Microbiol. 74: 1100–1107.
Love, M. I. et al. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. – Genome Biol. 15: 550.
McDonald, D. et al. 2012. The biological observation matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. – GigaScience 1: 7.
McKenna, P. et al. 2008. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. – PLoS Pathog. 4: e20.
McMurdie, P. J. and Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. – PLoS One 8: e61217.
Menke, S. et al. 2018. Data from: Impact of winter enclosures on the gut bacterial microbiota of red deer in the Bavarian Forest National Park. – Dryad Digital Repository, <http://dx.doi.org/10.5061/dryad.7r22vb1>.
Miller, R. et al. 2003. Evaluation of the influence of supplemental feeding of white-tailed deer (*Odocoileus virginianus*) on the prevalence of bovine tuberculosis in the Michigan wild deer population. – J. Wildl. Dis. 39: 84–95.
Möst, L. et al. 2015. Creating a landscape of management: unintended effects on the variation of browsing pressure in a national park. – For. Ecol. Manage. 338: 46–56.
Ossi, F. et al. 2017. Plastic response by a small cervid to supplemental feeding in winter across a wide environmental gradient. – Ecosphere 8: e01629.
Pellerin, M. et al. 2016. Complementary endozoochorous long-distance seed dispersal by three native herbivorous ungulates in Europe. – Basic Appl. Ecol. 17: 321–332.
Peng, K. et al. 2018. Condensed tannins affect bacterial and fungal microbiomes and mycotoxin production during ensiling and upon aerobic exposure. – Appl. Environ. Microbiol. 84: e02274-17.
Peterson, C. and Messmer, T. A. 2007. Effects of winter-feeding on mule deer in northern Utah. – J. Wildl. Manage. 71: 1440–1445.
Price, M. N. et al. 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. – PLoS One 5: e9490.
Putman, R. J. and Staines, B. W. 2004. Supplementary winter feeding of wild red deer *Cervus elaphus* in Europe and North America: justifications, feeding practice and effectiveness. – Mamm. Rev. 34: 285–306.
Rainey, F. 2009. Family VIII. *Ruminococcaceae* fam. nov. Bergey's manual of systematic bacteriology. – Springer.
Rooney, T. P. and Waller, D. M. 2003. Direct and indirect effects of white-tailed deer in forest ecosystems. – For. Ecol. Manage. 181: 165–176.
Sahlsten, J. et al. 2010. Can supplementary feeding be used to redistribute moose *Alces alces*? – Wildl. Biol. 16: 85–92.
Santín-Durán, M. et al. 2004. Abomasal parasites in wild sympatric cervids, red deer, *Cervus elaphu* and fallow deer, *Dama dama*, from three localities across central and western

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Spain: relationship to host density and park management. – J. Parasitol. 90: 1378–1386.
Schaller, M. J. 2007. Forests and wildlife management in Germany – a mini-review. – Eurasian J. For. Res. 10: 59–70.
Selva Fernández, N. 2004. The role of scavenging in the predator community of Bialowieza primeval forest: Papel de la necrofagia en la comunidad de predadores del bosque primigenio de Bialowieza (Polonia). – PhD thesis, Univ. of Sevilla.
Soest, P. J. V. 1994. Nutritional ecology of the ruminant. – Cornell Univ. Press.
Somerfield, P. 2008. Identification of the Bray–Curtis similarity index: comment on Yoshioka (2008). – Mar. Ecol. Progr. Ser. 372: 303–306.
Thomason, C. A. et al. 2017. Resident microbiome disruption with antibiotics enhances virulence of a colonizing pathogen. – Sci. Rep. 7: 16177.
Tohno, M. et al. 2016. *Paenibacillus silagei* sp. nov. isolated from corn silage. – Int. J. Syst. Evol. Microbiol. 66: 3873–3877.
Tremblay, J.-P. et al. 2007. Density-related effects of deer browsing on the regeneration dynamics of boreal forests. – J. Appl. Ecol. 44: 552–562.
Walke, J. B. and Belden, L. K. 2016. Harnessing the microbiome to prevent fungal infections: lessons from amphibians. – PLoS Pathog. 12: e1005796.
Wasimuddin et al. 2017. Gut microbiomes of free-ranging and captive Namibian cheetahs: diversity, putative functions and occurrence of potential pathogens. – Mol. Ecol. 26: 5515–5527.
Wasimuddin et al. 2018. Astrovirus infections induce age-dependent dysbiosis in gut microbiomes of bats. – ISME J. 12: 2883–2893.
Zhao, L. et al. 2015. Effects of nitrate addition on rumen fermentation, bacterial biodiversity and abundance. – Asian-Australasian J. Anim. Sci. 28: 1433–1441.
Zhu, L. et al. 2011. Evidence of cellulose metabolism by the giant panda gut microbiome. – Proc. Natl Acad. Sci. USA 108: 17714–17719.

Supplementary material (aavailabel online as Appendix wlb-00503 at <www.wildlifebiology.org/appendix/wlb-00503>). Appendix 1

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