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# Comparable response of wild rodent gut microbiome to anthropogenic habitat contamination

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## Abstract

Species identity is thought to dominate over environment in shaping wild rodent gut microbiota, but it remains unknown whether the responses of host gut microbiota to shared anthropogenic habitat impacts are species-specific or if the general gut microbiota response is similar across host species. Here, we compare the influence of exposure to radionuclide contamination on the gut microbiota of four wild mouse species: *Apodemus flavicollis*, *A. sylvaticus*, *A. speciosus* and *A. argenteus*. Building on the evidence that radiation impacts bank vole (*Myodes glareolus*) gut microbiota, we hypothesized that radiation exposure has a general impact on rodent gut microbiota. Because we sampled ( $n = 288$ ) two species pairs of *Apodemus* mice that occur in sympatry in habitats affected by the Chernobyl and Fukushima nuclear accidents, these comparisons provide an opportunity for a general assessment of the effects of exposure to environmental contamination (radionuclides) on gut microbiota across host phylogeny and geographical areas. In general agreement with our hypothesis, analyses of bacterial 16S rRNA gene sequences revealed that radiation exposure alters the gut microbiota composition and structure in three of the four species of *Apodemus* mice. The notable lack of an association between the gut microbiota and soil radionuclide contamination in one mouse species from Fukushima (*A. argenteus*) probably reflects host “radiation escape” through its unique tree-dwelling lifestyle. The finding that host ecology can modulate effects of radiation exposure offers an interesting counterpoint for future analyses into effects of radiation or any other toxic exposure on host and its associated microbiota. Our data show that exposure to radionuclide contamination is linked to comparable gut microbiota responses across multiple species of rodents.

## KEYWORDS

anthropogenic disturbance, environmental stress, gut microbiome, ionizing radiation, pollution

## 1 | INTRODUCTION

Gut microbiota provide many essential services to their animal hosts, including provision of diverse metabolites and nutrients, and protection against pathogens and toxic compounds (Parfrey et al., 2018; Suzuki, 2017). As persistence of these functions impact host health, variation of the gut microbiota composition holds importance for determining animals' adaptive potential in the face of environmental change (Alberdi et al., 2016). Despite such importance, responses of host-associated microbiota to environmental perturbations, particularly derived from human activity, remain largely unknown. At least partly, this is due to the lack of detailed characterization of microbial communities associated with animals in their natural habitat, where many anthropogenic stressors are now commonplace (Carthey et al., 2019; Rocca et al., 2019; Trevelline et al., 2019).

Wild animals living in sympatry can be expected to share exposures to common environmental conditions. These include, for example, persistent contaminants (e.g., xenobiotics, metals, radionuclides, microplastics) distributed due to their deliberate use or accidental release to the environment. Although a number of studies have shown that exposure to a variety of such contaminants can perturb gut microbiota diversity and composition (Chassaing et al., 2015; Goudarzi et al., 2016; Jin et al., 2019; Richardson et al., 2018), these studies were conducted with laboratory animals only (e.g., mice and rats) and lack broader ecological reality. In wild mammals, species identity is thought to dominate over environment in shaping gut microbiota (Knowles et al., 2019), and yet in many taxa (e.g., rodents, primates, other mammals) the strength of host species effects varies depending on features of host ecology including diet, lifestyle and space use strategy (Grond et al., 2019; Knowles et al., 2019; Moeller et al., 2013; Perofsky et al., 2019). Hence, it is unclear whether the responses of host gut microbiota to shared anthropogenic habitat impacts are species-specific (distinct responses) or if the general gut microbiota response is comparable (parallel responses) across host species.

Exposure to radionuclides presents diverse risks with overall negative impacts on animal health (Møller & Mousseau, 2006). Numerous human activities worldwide have contributed to radionuclide contamination of the environment through nuclear weapons tests, uranium mining and nuclear accidents (Lourenço et al., 2016). The largest releases of radionuclides into the environment occurred due to the accidents at Chernobyl and Fukushima nuclear power plants (NPPs) on April 26, 1986 and March 11, 2011, respectively. In response to the accidents, humans were evacuated from a 4,760-km<sup>2</sup> area in Ukraine (and partly in Belarus) and from a 1,150-km<sup>2</sup> area in Japan; these abandoned areas are often respectively referred to as the Chernobyl Exclusion Zone (CEZ) and the Fukushima Evacuation/Exclusion Zone (FEZ). Due to environmental contamination by radionuclides with long half-lives (principally <sup>137</sup>Cs, 30 years; and <sup>90</sup>Sr, 29 years) access to these evacuation zones remains severely restricted for human habitation (Beresford et al., 2020; Harada et al., 2014), but not wildlife. Animals inhabiting the

CEZ and FEZ thus provide the best-studied models of the biological impacts of exposure to radionuclide contamination.

A number of ecological studies have documented negative effects of exposure to radionuclides in wildlife from Chernobyl and Fukushima (reviewed by Lourenço et al., 2016; Møller & Mousseau, 2006; Mousseau & Møller, 2014; Strand et al., 2017). For example, radionuclide contamination at both accident sites has been associated with negative health impacts, evident as an apparent increase in genetic damage, oxidative stress (Bonisoli-Alquati et al., 2010; Boratyński et al., 2014; Einor et al., 2016; Hiyama et al., 2012; Lourenço et al., 2016) and various developmental abnormalities (Hayama et al., 2017; Møller et al., 2011), with fitness consequences and population-level effects (Mappes et al., 2019). Only a few studies have attempted a direct hypothesis-driven comparison of the biological effects of radionuclide contamination at the two accident sites; a consistent decline in the abundance of birds with increasing levels of radionuclide contamination (Møller et al., 2012), and little notable effects of radiation on the abundance of large mammals (Lyons et al., 2020; Webster et al., 2016) were reported at both the CEZ and the FEZ.

Despite the efforts in surveying Chernobyl and Fukushima wildlife, only a few studies have quantified the effects of radionuclide contamination on host-associated microbiota; the available studies were all conducted in the CEZ only. For example, the total cultivable bacterial loads from feathers of birds nesting at Chernobyl were negatively correlated with radionuclide contamination levels (Czirják et al., 2010; Ruiz-González et al., 2016). Similarly, the diversity of the gut microbiota of earthworms collected from contaminated areas in the CEZ was reduced compared with uncontaminated control areas (but see effects of soil pH) (Newbold et al., 2019). In contrast, results from the only studied mammal, the bank vole *Myodes glareolus*, indicate that radiation exposure does not reduce estimates of gut or skin microbiota diversity (Lavrinenko, Mappes, et al., 2018; Lavrinenko, Tukalenko, et al., 2018). Instead, exposure to radionuclides was associated with a major change in bank vole gut microbiota composition, notable as a reduction in the proportion of Bacteroidetes and an increase in Firmicutes (Lavrinenko, Mappes, et al., 2018). As a result, bank voles inhabiting areas contaminated by radionuclides can be identified by their distinct gut microbiota. Importantly, it remains unknown whether such gut microbiota responses to radiation exposure are specific to bank voles or represent a more general pattern similar across other wild rodents from Chernobyl, or even other nuclear accident sites similarly contaminated with radionuclides, such as Fukushima.

To obtain a general assessment of the effects of exposure to radionuclide contamination upon rodent gut microbiota, we analysed faecal samples ( $n = 288$ ) from two pairs of mouse species (*Apodemus flavicollis*, *A. sylvaticus*, *A. speciosus*, *A. argenteus*) that occur in sympatry in habitats surrounding the Chernobyl and Fukushima nuclear accident sites. We hypothesized that radiation exposure has general impact on rodent gut microbiota, and thus similar levels of exposure would (i) alter gut microbiota composition in all mouse species, with the overall patterns comparable to those reported in bank voles

living under chronic radiation exposure (Lavrinenko, Mappes, et al., 2018). Specifically, we predicted (ii) that such altered community composition would be characterized by an increase in the ratio of the Firmicutes to Bacteroidetes phyla (F:B), which could possibly act as a “biomarker” of exposure to radiation; (iii) that exposure to radiation also would have little notable effect on the diversity of mouse gut microbiota; and (iv) that exposure to radiation would select for distinct gut microbiota structure in wild mice as found for bank voles.

## 2 | MATERIALS AND METHODS

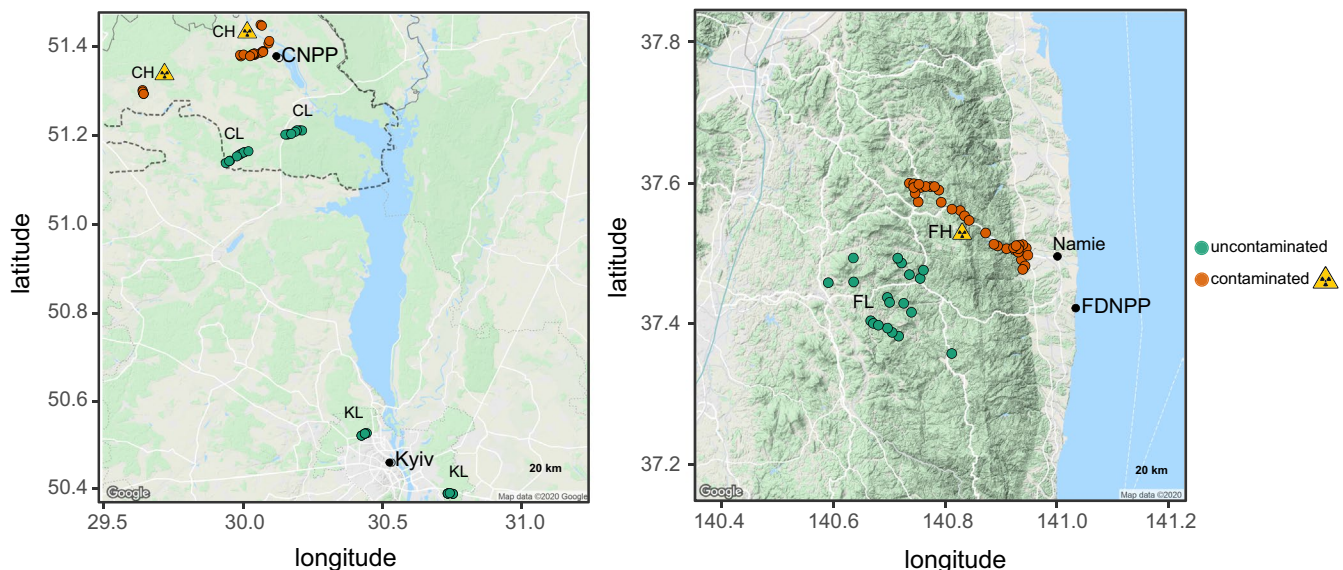
### 2.1 | Study species

*Apodemus flavicollis* and *A. sylvaticus* occur in sympatry throughout much of the Western Palearctic region, while *A. speciosus* and *A. argenteus* are also sympatric, but endemic to Japan (Michaux et al., 2005; Suzuki et al., 2008). These pairs of *Apodemus* species are also among the most common mammals in forested areas around the respective Chernobyl and Fukushima accident sites (Baker et al., 1996; Beresford et al., 2008; Kubota et al., 2015). These mice are typical granivorous rodents whose diets are largely dominated by mast seeds (e.g., acorns and other nuts) or weed seeds, but sometimes include vegetative plant material, invertebrates and/or fungi (Butet & Delettre, 2011). *A. flavicollis* and *A. sylvaticus* have similar ecology and diets (Knowles et al., 2019; Michaux et al., 2005; Ozaki et al., 2018), whereas despite overlapping habitats, soil-dwelling *A. speciosus* and tree-dwelling *A. argenteus* segregate vertically and differ in dietary preferences (specialist and generalist, respectively) (Oka, 1992; Sato et al., 2018).

### 2.2 | Study design

Trapping locations were designed so that animals had experienced low-dose rate radiation exposure ( $4\text{--}40\ \mu\text{Gy hr}^{-1}$ ) relevant for assessment of potential impacts of radiation on wildlife (Real & Garnier-Laplace, 2019). We assigned trapping locations as either (i) contaminated or (ii) uncontaminated (Figure 1; hereafter “treatment”) based on a contrast in the ambient radiation dose rates (radionuclide contamination of the environment, measured at 1 cm above the ground using a hand-held Geiger counter (Table 1; Supporting Information). Hence, contaminated areas within the CEZ and FEZ (Chernobyl High, CH; Fukushima High, FH) had significantly higher levels of radiation ( $\chi^2 = 43.47$ ,  $df = 2$ ,  $p < .001$ , Kruskal-Wallis test and  $W = 0$ ,  $p < .001$ , Wilcoxon rank-sum test, respectively) compared with uncontaminated areas (Chernobyl Low, CL; Kyiv Low, KL; Fukushima Low, FL).

Mice were sampled from mixed woodland areas with similar habitats across both locations of high and low radionuclide contamination. In the CEZ, we sampled replicate (spatially separated) locations within each treatment to deconfound effects of exposure to radiation from other potential environmental factors specific to a certain location. Treatment-level replicate sites in Ukraine were separated by 10–80 km (Figure 1), which exceeds seasonal dispersal capabilities of the study species ( $<1.5$  km for adults, Stradiotto et al., 2009). In Japan, widespread agriculture around the FEZ prevented such replication. Hence, to minimize site-specific effects we set a large number ( $n = 32$ ) of trapping locations across a ~25-km transect of the most contaminated “difficult-to-return” zone (Harada et al., 2014) and also sampled from diverse locations in uncontaminated areas ( $n = 19$ ) (Figure 1).



**FIGURE 1** Map of the study areas with mice trapping locations in Ukraine (left) and Japan (right) shown by points. Areas contaminated (CH, FH in orange) and uncontaminated (CL, KL, FL, green) with radionuclides within the Chernobyl and Fukushima Exclusion Zones (CEZ/FEZ) are shown. Dashed line represents the border around the CEZ in Ukraine (area of ~2,050 km<sup>2</sup>). The border around the FEZ in Japan is not shown; mice trapping locations in contaminated areas within the FEZ were located within the most contaminated “difficult-to-return” zone. The figure was created using the GGMAP version 3.0.0 package in R

**TABLE 1** Mean estimates of the internal, external and total radiation dose rates for each of the four sampled mouse species ( $n = 288$ ) inhabiting areas contaminated with radionuclides (CH and FH) and uncontaminated areas (CL, KL, FL) in Ukraine and Japan

Species	Treatment group	Ambient radiation dose rate (mGy day <sup>-1</sup> )	Internal dose rate (mGy day <sup>-1</sup> )	External dose rate (mGy day <sup>-1</sup> )	Total dose rate (mGy day <sup>-1</sup> )	External dose in total (%)
<i>Apodemus flavicollis</i>	CH, $n = 60$	0.546	0.050	0.498	0.548	90.8
	CL, $n = 31$	0.005	0.001	0.005	0.006	85.1
	KL, $n = 36$	0.005	0.001	0.005	0.006	93.1
<i>Apodemus sylvaticus</i>	CH, $n = 14$	0.546	0.048	0.313	0.361	86.7
	CL, $n = 11$	0.005	0.002	0.006	0.007	78.6
	KL, $n = 4$	0.005	<0.001	0.007	0.007	100.0
<i>Apodemus argenteus</i>	FH, $n = 28$	0.205	0.012	0.233	0.245	95.1
	FL, $n = 25$	0.013	0.001	0.013	0.014	94.9
<i>Apodemus speciosus</i>	FH, $n = 54$	0.205	0.011	0.174	0.185	94.2
	FL, $n = 25$	0.013	0.001	0.017	0.018	96.7

## 2.3 | Mouse trapping and sampling

Mice were caught by live trapping (Lavrinienko, Mappes, et al., 2018). Briefly, *A. flavicollis* ( $n = 127$ ) and *A. sylvaticus* ( $n = 29$ ) were caught at 59 locations around northern Ukraine and within the CEZ (Figure 1) during June 12 to July 29, 2016. At each location, 16 Ugglan Special2 (Grahnb) traps baited with sunflower seeds and potato were placed in a  $4 \times 4$  grid, with an intertrap distance of 20 m. Traps were deployed in the late afternoon and animals were collected early the following morning. In Japan, *A. speciosus* ( $n = 79$ ) and *A. argenteus* ( $n = 53$ ) were caught at 51 locations using either Ugglan or Sherman live traps (baited with sunflower seeds and apple) during September 8–19, 2015 (Figure 1). In the FEZ, 12 traps were placed in three small ( $10 \times 10$  m) quadrants instead of larger grids. Given the home ranges (<0.5 ha) of the species studied (Godsall et al., 2014; Oka, 1992; Stradiotto et al., 2009), in both study sites the minimum distance between trapping locations was at least 500 m.

Captured animals were transported to field laboratories for species' identification, and classification of sex. We also recorded body mass, head width and maturity (juvenile, subadult, adult) (Table S1). Body condition index (BCI) was calculated for each individual as the standardized residual values from a linear regression of weight against head width (Schulte-Hostedde et al., 2001): a positive BCI is indicative of a better condition and greater energy reserves (Schulte-Hostedde et al., 2001). Animals were killed by cervical dislocation and preserved at  $-20^{\circ}\text{C}$  until dissection, when an  $\sim 2$ -cm section of the distal colon was removed to collect faecal material (kept frozen at  $-20^{\circ}\text{C}$  for  $\sim 10$  days, and stored at  $-80^{\circ}\text{C}$  until DNA extraction). To avoid potential batch effects and systematic bias, we randomized samples (by host species and treatment) during DNA extraction, library preparation and sequencing work. All samples were always transported and stored together.

## 2.4 | DNA extraction and sequencing

Total DNA was extracted from faecal samples ( $n = 288$ ) using a PowerFecal DNA Isolation kit (MOBIO Laboratories) following the

manufacturer's instructions for the manual single tube-based extraction method. Potential contamination of samples by reagents or the wider laboratory environment was limited following guidelines for sequence-based analyses of microbial communities (Eisenhofer et al., 2019). DNA extractions were performed within a laminar flow hood using aseptic techniques (e.g., surface sterilization, sterile plasticware, aerosol barrier filter tips). The same DNA extraction kit batch was used for all the samples. In these settings, we did not use "blank" sterile water controls at the DNA extraction step. All library preparation and sequencing work was performed at the Institute for Molecular Medicine Finland (FIMM, University of Helsinki) ([www.fimm.fi](http://www.fimm.fi)). To control for potential contamination, negative controls were included during library preparation work at FIMM. Briefly, the V4 variable region ( $\sim 254$  bp) of the 16S ribosomal RNA (rRNA) locus was amplified using the original 515F/806R primer pair (Caporaso et al., 2011). We used primers with built-in heterogeneity spacers to ensure balanced nucleotide diversity of sequencing libraries for optimal Illumina MiSeq run performance (Table S2). Amplification was done in a multiplex PCR (polymerase chain reaction) with locus-specific primers carrying Illumina adapter tails and Illumina P5/P7 sequences (every sample had unique index combination). PCR conditions were:  $95^{\circ}\text{C}$  for 5 min, followed by 27 cycles at  $95^{\circ}\text{C}$  for 30 s, at  $62^{\circ}\text{C}$  for 1.5 min and at  $72^{\circ}\text{C}$  for 30 s; and then  $68^{\circ}\text{C}$  for 10 min. PCR products were pooled in equal volumes and purified with an Agencourt AMPure XP PCR Purification kit (Beckman Coulter) using  $0.8\times$  volume of beads compared to the library pool volume. The final library was quantified using an Agilent High Sensitivity DNA Kit (Agilent Technologies) on an Agilent 2100 Bioanalyzer. Libraries were sequenced on an Illumina MiSeq to provide 250-bp paired-end (PE) reads. The negative controls were not sequenced as they did not generate any PCR product.

## 2.5 | Read data processing

Read data were demultiplexed, and adapters and spacers were removed by FIMM. Sixteen samples were not analysed due to a low



number (<600) of reads per sample (Table S1). The PE sequences (total =13,032,540, mean =47,913, range 12,257–286,271) for the remaining 272 samples (*A. flavicollis*  $n = 115$ , *A. sylvaticus*  $n = 27$ , *A. speciosus*  $n = 77$ , *A. argenteus*  $n = 53$ ) were processed using DADA2 (Callahan et al., 2016) in QIIME2 version 2019.4 (Bolyen et al., 2019). Briefly, reads were truncated at the 3' end to remove low-quality base calls (<Q25, forward reads at 243 bp and reverse at 176 bp). Primer sequences were trimmed from the 5' end of all reads (DADA2 *trim-left-f* 19, *trim-left-r* 20) (Caporaso et al., 2011), after which data were de-noised and dereplicated and putative chimeric sequences removed using default parameters in DADA2 (Callahan et al., 2016). After quality control, the feature-table retained 11,930,717 sequences, with 4,510 amplicon sequence variants (ASVs) (Callahan et al., 2016). Taxonomy was assigned using a naïve Bayes classifier that has been pretrained on the Greengenes version 13\_8 16S rRNA database (with sequences trimmed to the V4 region bounded by 515F/806R primers, and clustered at 99% identity) (Bokulich et al., 2018). The ASVs represented by fewer than 10 reads across all samples were removed in order to filter out potential errors and spurious artefacts (such a filtering strategy has little impact on the alpha- and beta-diversity estimates, Figure S1; see test details in Supporting Information). This step left 11,927,082 sequences (mean =43,849, range 10,553–263,628 sequences per sample) and 3,760 ASVs. These data were rarefied to 10,553 sequences per sample (3,731 ASVs) and this normalized (Weiss et al., 2017) feature-table was used for subsequent analyses.

## 2.6 | Radiation dose estimation

The total radiation exposure of each mouse was determined from the sum of the external (from surrounding environment) and internal (from ingested particles) radiation dose rates (Table 1; Supporting Information). External radiation exposure (absorbed dose) of mice was estimated from the ambient radiation dose rate levels (radionuclide contamination of the environment, typically measured using a hand-held Geiger counter) at trapping locations (Table S1). This approach has been experimentally verified using implanted thermoluminescent dosimeters (TLDs) that directly measure absorbed external radiation dose rate in vivo (Beresford et al., 2008; Chesser et al., 2000; Lavrinienko et al., 2020). We also conducted a capture–mark–recapture pilot study and implanted TLDs ( $n = 10$ , CHP Dosimetry) in *A. flavicollis* mice from contaminated and uncontaminated areas within the CEZ (Supporting Information). While external dose readings for three recaptured individuals conform closely (Table S3) to those reported in other studies (Beresford et al., 2008), a low mice recapture rate (<25%) prevented such trials in other species. Internal radiation exposure of all mice (whole-body radiocaesium burden) was estimated using  $\gamma$ -spectrometry (SAM 940; Berkeley Nucleonics) (Table 1; Supporting Information).

Total radiation exposure differed somewhat between species (Table 1), although consistently, most (~78% to >96%) of the total radiation exposure in all mice is derived from external sources; that is, animals are exposed to radiation simply by living in a contaminated

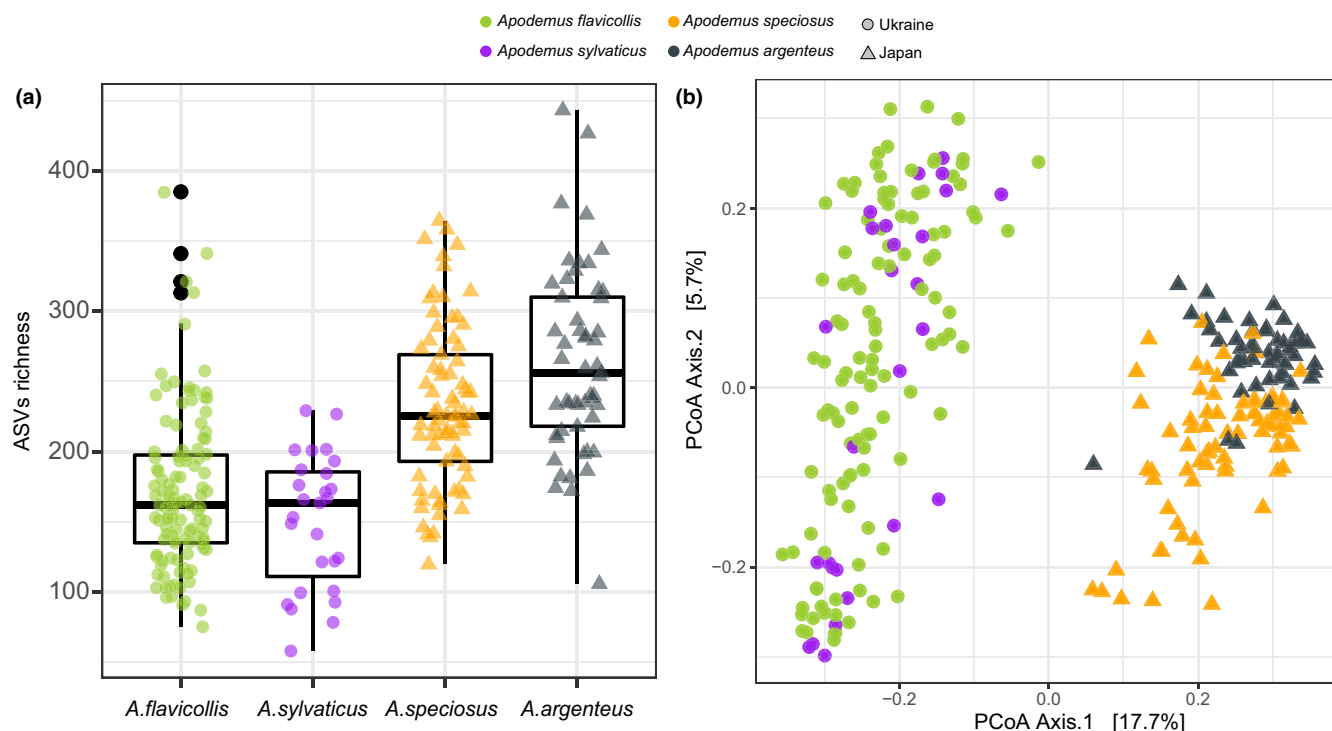
area (Beresford et al., 2020; Kubota et al., 2015; Onuma et al., 2020). The average total radiation dose rates for mice captured from contaminated areas were 0.51 mGy day<sup>-1</sup> in CEZ and 0.21 mGy day<sup>-1</sup> in FEZ, which is one to two orders of magnitude more than exposure received by mice from uncontaminated areas (Table 1). These radiation doses are similar to those reported in other studies of rodents from the Chernobyl and Fukushima accident sites (Beresford et al., 2020; Kubota et al., 2015; Onuma et al., 2020). For context, the dose rates in *A. flavicollis* and *A. speciosus* are equivalent to about two to four chest radiography scans (~0.10–0.15 mGy) each day (Baker et al., 2017; Brenner & Hall, 2007). The implication of the radiation dosimetry data is that mice inhabiting the contaminated (CH, FH), but not uncontaminated (CL, KL, FL), areas in Ukraine and in Japan experience significant chronic radiation exposure (Table 1).

## 2.7 | Statistical analyses

Statistical analyses were performed using R version 3.6.1 (R Core Team, 2019). We analysed alpha-diversity by calculating ASV richness, Faith's phylogenetic diversity and Shannon index for each sample. Significant differences between groups (i.e., host species, treatment and sex) were determined using either Wilcoxon rank-sum or Kruskal–Wallis tests (when more than two groups were compared), followed by a *post hoc* Dunn test with a Benjamini–Hochberg false discovery rate (FDR) correction for multiple comparisons in the R package DUNN.TEST (version 1.3.5) (Dinno, 2017). We examined potential associations between continuous metadata variables (e.g., total radiation dose rate, body condition) and mouse gut microbiota alpha-diversity estimates using Spearman's rank correlations.

To examine differences in beta-diversity between treatments we calculated pairwise sample dissimilarities using four beta-diversity metrics (Bray–Curtis dissimilarity, Jaccard similarity index, unweighted and weighted UniFrac distances) in the R package PHYLOSEQ (version 1.30.0) (McMurdie & Holmes, 2013). Sample clustering patterns (based on the Bray–Curtis dissimilarity) were visualized by principal coordinates analysis (PCoA) (McMurdie & Holmes, 2013). We tested for significant differences in sample grouping with a permutational analysis of variance (PERMANOVA, 999 permutations) using the *adonis* function in the R package VEGAN (Oksanen et al., 2019). Dispersion tests using *permdisp* in QIIME2 were used to assess whether significant treatment effects could be influenced by differences in group dispersion rather than centroids (Anderson, 2001; Bolyen et al., 2019).

To identify which ASVs significantly differed in abundance between treatments, we used the permuted mean difference tests with 10,000 permutations and discrete FDR correction at alpha 0.1, using DS-FDR (version 0.0.2) (Jiang et al., 2017). In addition, the RANDOM FOREST (RF) machine-learning algorithm (Breiman, 2001) was used to determine the accuracy with which samples could be assigned to one of the study sites (Ukraine vs. Japan), and host species, based on gut microbiota community composition. Models were built using the Q2-SAMPLE-CLASSIFIER (Bokulich et al., 2018), with 10,000 trees and a five-fold cross-validation scheme. Input samples were randomly



**FIGURE 2** Interspecific variation in wild mouse gut microbiota diversity and structure. Measures of (a) alpha diversity based on the ASV richness, and (b) beta diversity based on the Bray–Curtis dissimilarity for the gut microbiota of four mouse species inhabiting areas surrounding either the Chernobyl, Ukraine (*Apodemus flavicollis*, *A. sylvaticus*), or Fukushima, Japan (*A. speciosus*, *A. argenteus*), nuclear accident sites. (a) Box-and-whisker plots represent the median and interquartile range of ASV richness. (b) Each point represents a single sample, while shape indicates the country of origin. Samples are coloured according to the host species

split into training and test sets at a 4:1 ratio, and automatic feature (ASV) selection and parameter optimization steps were enabled to tune the model. ASVs that maximize predictive accuracy were identified and assigned an importance score using a cross-validated recursive feature elimination procedure (Bokulich et al., 2018).

Additionally, we re-analysed the data set (e.g., EBI accession no. ERP104266) from the study that examined the gut microbiota of bank voles (*Myodes glareolus*) exposed to radionuclide contamination in Chernobyl (Lavrinenko, Mappes, et al., 2018). We directly compared the generality of radiation-associated changes in the bank vole gut microbiota (e.g., differences in alpha- and beta-diversity between treatments) to the patterns observed in *A. flavicollis* and *A. sylvaticus*; thus, we examined three species that occur in sympatry in habitats affected by the Chernobyl nuclear accident. All details about data processing and results provided in the Supporting Information.

### 3 | RESULTS

#### 3.1 | Wild mouse gut microbiota composition

We identified 3,731 ASVs from 15 bacterial phyla in the gut microbiota of four species of mice ( $n = 272$ ) inhabiting areas surrounding the Chernobyl and Fukushima nuclear accident sites (Table S4; Figure 1).

In all species of mice, three bacterial phyla accounted for >97% of the gut microbiota community, Bacteroidetes (mean = 67%), Firmicutes (27%) and Proteobacteria (3%), albeit with different proportions among host species (Table S4). Bacteroidetes were dominated by members of the S24-7 family (>84% of Bacteroidetes, mean = 57% of total community), while Firmicutes mainly comprised three families: *Lachnospiraceae* (7%), *Lactobacillaceae* (6.5%) and *Ruminococcaceae* (6%). This community composition is typical for the gut microbiota of wild mice from the genus *Apodemus* (Knowles et al., 2019; Maurice et al., 2015).

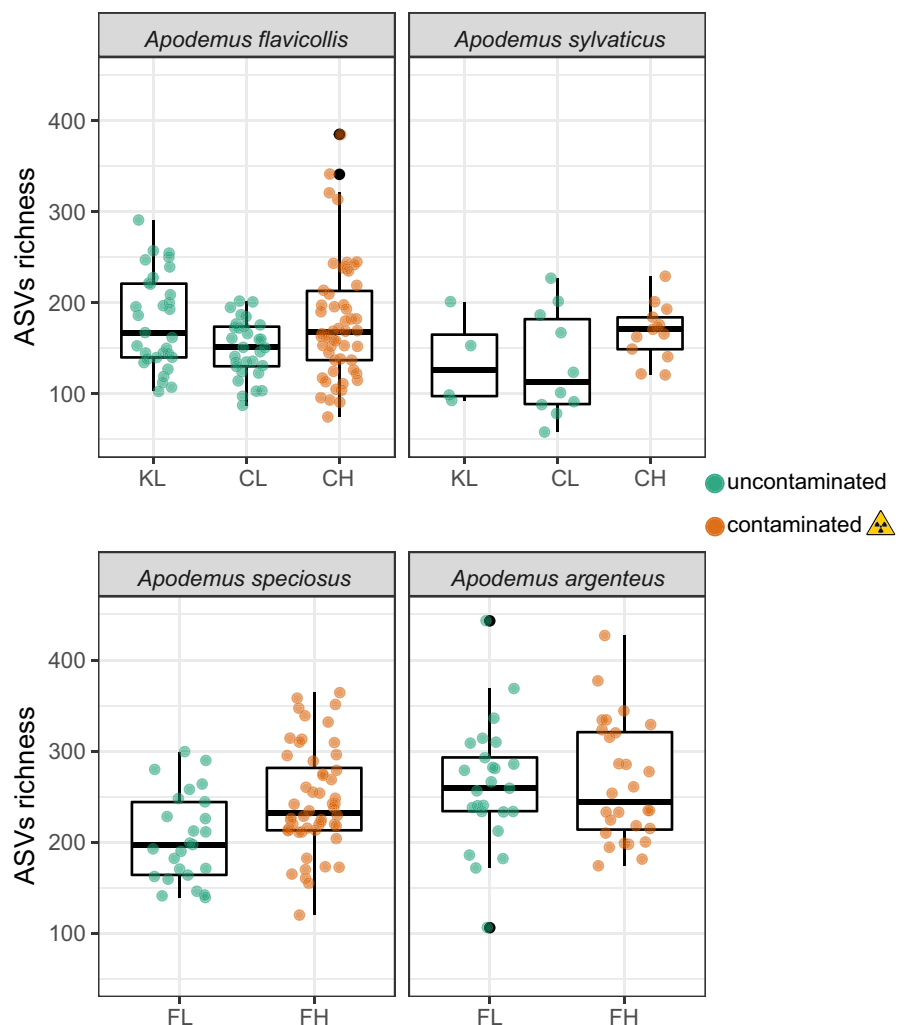
#### 3.2 | Interspecific variation in wild mouse gut microbiota

When compared across species, irrespective of radionuclide contamination, *Apodemus* mice harbour distinct gut microbiota, both between Europe and Japan and in sympatry. For example, species from Japan (*A. speciosus* and *A. argenteus*) were characterized by significantly ( $\chi^2 = 96.27$ ,  $df = 3$ ,  $p < .001$ , Kruskal–Wallis test for ASV richness) more diverse gut microbiota than their congeners in Ukraine (*A. flavicollis*, *A. sylvaticus*) (Figure 2a). The country of origin (i.e., from Ukraine or Japan) accounted for ~17% of the variation in gut microbiota structure ( $F = 56.12$ ,  $R^2 = .17$ ,  $p < .001$ , PERMANOVA based on Bray–Curtis dissimilarity) between all

samples (Figure 2b; Table S5). Consistent with this observation, RF supervised learning models could correctly classify all samples from Ukraine and Japan (baseline accuracy 0.53, accuracy ratio 1.9) (Figure S2a). We also trained the RF classifier to identify host species; RF models assigned samples to respective mouse species with 90% accuracy (baseline 0.42, ratio 2.13) (see Table S6 for the top 20 most important ASVs). Notably, the predictive accuracy of this RF classifier decreased due to misclassifications only between samples from *A. flavicollis* and *A. sylvaticus* hosts (Figure S2b,c). Hence, of the sympatric species pairs, *A. flavicollis* and *A. sylvaticus* had a more similar gut microbiota compared with *A. speciosus* and *A. argenteus*. Indeed, *A. argenteus* harboured significantly ( $\chi^2 = 96.27$ ,  $df = 3$ ,  $p < .021$ , Kruskal–Wallis test for ASV richness) higher gut bacterial diversity compared with *A. speciosus*, but there was no significant difference in diversity of the gut microbiota of *A. flavicollis* and *A. sylvaticus* (Figure 2a). Moreover, while host identity was a significant predictor of gut microbiota profiles ( $F = 6.91$ ,  $R^2 = .04$ ,  $p < .001$ , PERMANOVA based on Bray–Curtis dissimilarity), only *A. speciosus* and *A. argenteus*, and not the *A. flavicollis* and *A. sylvaticus* pair, exhibited an apparent species-specific samples clustering pattern (Figure 2b; see Table S5 for PERMANOVA results testing for species differences in each country).

### 3.3 | The effects of radiation on mouse gut microbial community diversity

Exposure to environmental radionuclides has little notable effect on the alpha diversity of wild mouse gut microbiota. Thus, gut microbiota diversity was not affected by radioactivity ( $\chi^2 = 5.18$ ,  $df = 2$ ,  $p > .075$  and  $\chi^2 = 2.65$ ,  $df = 2$ ,  $p > .265$ , Kruskal–Wallis tests for ASV richness in *A. flavicollis* and *A. sylvaticus*, respectively;  $W = 358$ ,  $p > .886$ , Wilcoxon rank-sum test for ASV richness in *A. argenteus*) in any of the mouse species except *A. speciosus* (Figure 3). That the gut microbiota diversity did not differ significantly between treatments was also consistent in bank voles ( $\chi^2 = 2.66$ ,  $df = 2$ ,  $p > .264$ , Kruskal–Wallis test for ASV richness; see Figure S3a). In *A. speciosus*, animals from contaminated areas (FH) had significantly ( $W = 382$ ,  $p < .004$ , Wilcoxon rank-sum test for ASV richness) higher alpha diversity compared with animals from uncontaminated (FL) areas (Figure 3; Figure S4). The significant positive trend seen in *A. speciosus*, but not the other host species, was also retained when total radiation dose rate for individual mice was used as a continuous explanatory variable ( $\rho = 0.26$ ,  $p < .020$ , Spearman's correlation for ASV richness; Figure S5, Table S7). These patterns in gut microbiota diversity were consistent across all three alpha diversity measures (e.g., ASV



**FIGURE 3** Measures of alpha diversity for the gut microbiota of four mouse species inhabiting areas that differ in levels of radionuclide contamination. Box-and-whisker plots represent the median and interquartile range of ASV richness. Each box plot represents samples from areas contaminated with radionuclides (CH, FH) or uncontaminated (KL, CL, FL) surrounding either the Chernobyl (*Apodemus flavicollis*, *A. sylvaticus*) or Fukushima (*A. speciosus*, *A. argenteus*) nuclear accident sites



richness, Faith's phylogenetic diversity and Shannon index). None of the host-related factors, such as sex or body condition, were associated with gut microbiota alpha diversity estimates in all species of mouse except *A. argenteus* (consistent across all three alpha-diversity measures; Figures S6 and S7, Table S7). This is because, in *A. argenteus*, host body condition exhibited a weak positive association ( $\rho = 0.28$ ,  $p < .042$ , Spearman's correlation for ASV richness; but nonsignificant for Shannon index, see Table S7) with gut microbiota alpha diversity (Figure S7); this latter association was not related to radiation exposure as body condition was not correlated with total radiation dose in any of the sampled mice (Figure S8).

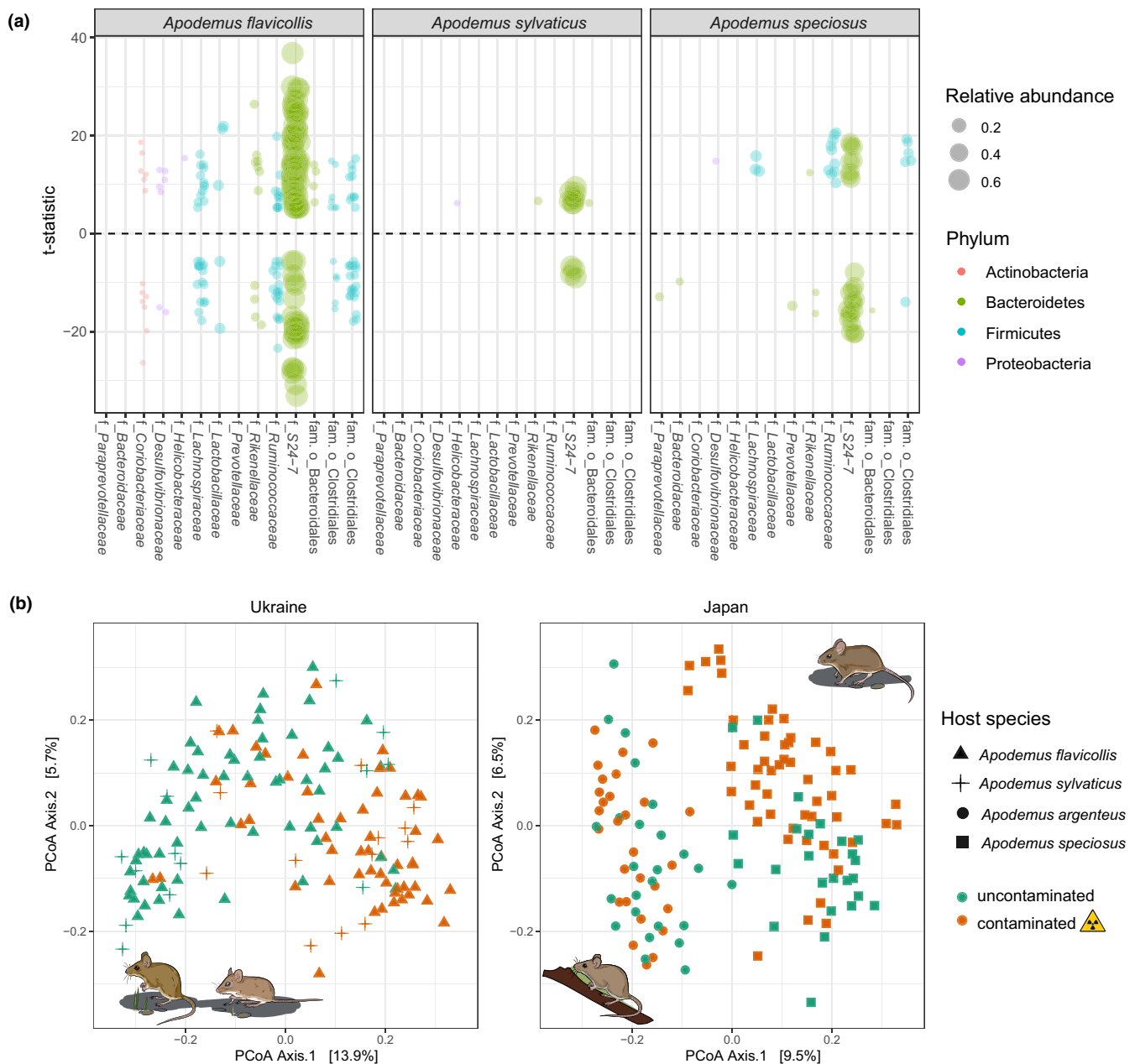
### 3.4 | Radiation-associated differences in mouse gut microbiota composition

Radiation exposure was associated with altered gut microbiota composition in all species of mouse except *A. argenteus* ( $p < .05$ , permutation test with discrete FDR (dsFDR) correction; Figure 4a). We found that among the 305 bacterial ASVs (from taxa with  $>0.5\%$  mean relative abundance) that were differentially abundant between treatments in *A. flavicollis*, *A. sylvaticus* and *A. speciosus*, about half ( $n = 130$ ; 40%, 80% and 47% ASVs per species, respectively) of the ASVs were assigned to the Bacteroidetes family S24-7 (Table S8). Moreover, members of the S24-7 family were significantly ( $\chi^2 = 48.68$ ,  $df = 5$ ,  $p < .001$ , Kruskal-Wallis test) over-represented in the gut microbiota of mice from contaminated areas. This trend was consistent in two mouse species, *A. flavicollis* and *A. sylvaticus*, inhabiting the CEZ (Figure S9). The frequency of the S24-7 ASVs was similar ( $\chi^2 = 3.38$ ,  $df = 3$ ,  $p > .336$ , Kruskal-Wallis test) between treatments in both *A. speciosus* and *A. argenteus*. Interestingly, all but one of the 12 differentially abundant S24-7 ASVs in *A. sylvaticus* were identical to those ASVs that exhibited a significant change in proportion in relation to radiation in *A. flavicollis* (Table S8). Such parallel changes were observed also in a few other taxa (e.g., ASVs assigned to *Rikenellaceae*, *Helicobacteraceae* and Bacteroidales), suggesting similar responses to radiation in the gut microbiota of these two mouse species inhabiting the CEZ. That said, while no members of the Firmicutes were differentially abundant between treatments in *A. sylvaticus*, we identified 109 and 19 ASVs assigned to Firmicutes, which exhibit a significant ( $p < .05$ , permutation test with discrete FDR (dsFDR) correction) difference in relative abundance among contaminated and uncontaminated areas in *A. flavicollis* and *A. speciosus*, respectively (Figure 4a; Table S8). At the lower taxonomy levels, these ASVs were assigned to *Lactobacillaceae* ( $n = 5$  ASVs), *Ruminococcaceae* ( $n = 41$ ), *Lachnospiraceae* ( $n = 33$ ) and other families within the Clostridiales ( $n = 49$ ) (Table S8). Hence, members of the phyla Firmicutes and Bacteroidetes drive compositional changes in response to radiation exposure in wild mice. The high number of differentially abundant ASVs ( $n = 130$ ) relative to the Firmicutes families, higher overall diversity in contaminated areas, and consistent changes in two host species suggest members of the Bacteroidetes family S24-7 are particularly responsive to radiation exposure.

It is important to note that members of the S24-7, *Ruminococcaceae*, *Lachnospiraceae* and other Clostridiales were both negatively and positively affected (most taxa in ~1:1 ratio, but see 46 vs. 84 S24-7 ASVs, respectively) by radiation exposure (Figure 4a; Table S8). Hence, an effect of radiation was not apparent ( $\chi^2 = 1.18$ ,  $df = 2$ ,  $p > .554$  and  $\chi^2 = 1.66$ ,  $df = 2$ ,  $p > .435$ , Kruskal-Wallis tests for *A. flavicollis* and *A. sylvaticus*, respectively;  $W = 277$ ,  $p > .196$ , Wilcoxon rank-sum test for *A. argenteus*) as a change in the ratio of the Firmicutes to Bacteroidetes phyla (F:B). Indeed, only *A. speciosus* inhabiting contaminated (FH) areas were characterized by a significant ( $W = 349$ ,  $p < .001$ , Wilcoxon rank-sum test) increase in F:B ratio compared with FL areas (Figure S10). This implies that, contrary to our prediction, the F:B ratio is not a general biomarker of radiation exposure, because changes in the gut microbiota of mice inhabiting radioactively contaminated areas operate on the level of distinct ASVs. Hence, effects of radiation on mouse gut microbiota composition appear to be taxonomically widespread across many bacterial families, with members of each family both positively and negatively associated with radiation exposure.

### 3.5 | Radiation-associated differences in mouse gut microbiota structure

Variation in the mouse gut microbiota structure reinforces the patterns of community composition described above. The gut microbiota samples from contaminated with radionuclides and uncontaminated areas were clearly separated in the ordination space (Figure 4b, Bray-Curtis dissimilarity; see also Table S5) in three species of mouse (i.e., *A. flavicollis*, *A. sylvaticus* and *A. speciosus*); this pattern was also consistent in bank voles (see Figure S3b and Supporting Information for more details). Accordingly, gut microbiota significantly differed among radionuclide contamination treatments ( $p < .01$ , PERMANOVA based on Bray-Curtis dissimilarity), but not between sexes or due to variation in body mass, head width (but see *A. speciosus*) or maturity (Table S5, and Supporting Information for details on bank voles). While exposure to radionuclide contamination predicted gut microbiota structure in mice, within each treatment the total radiation dose rates explained little (1%–4%) additional variation in the gut microbiota profiles among sampled mice. These patterns were largely consistent across all four beta diversity metrics used (see Table S5). A notable exception to this again was *A. argenteus*, where host sex and maturity, but not radionuclide contamination treatment, made significant ( $p < .05$ , PERMANOVA based on Bray-Curtis dissimilarity) contributions to the total variation in the gut microbiota profiles (Figure 4b; Table S5). We found no significant ( $p > .05$ , PERMDISP) difference in dispersion between treatment groups, and thus the distinct gut microbiota profiles in *A. flavicollis*, *A. sylvaticus* and *A. speciosus* mice between treatments resulted from differences in group centroids. Hence, exposure to radionuclide contamination alters the gut microbiota structure in three out of the four species of *Apodemus* mice.



**FIGURE 4** Radiation-associated differences in mouse gut microbiota community composition and structure. (a) The 305 bacterial ASVs that were differentially abundant among radionuclide contamination “treatments” in mice inhabiting areas surrounding either the Chernobyl (*Apodemus flavicollis*, *A. sylvaticus*) or Fukushima (*A. speciosus*) nuclear accident sites. Data for three mouse species are shown as none of the ASVs were differentially abundant between treatments in *A. argenteus*. ASVs below the dashed line ( $t$ -statistic from the permutation test with dsFDR correction) are more abundant in samples from uncontaminated (KL, CL, FL) areas, while those above are more abundant in samples from contaminated (CH, FH) areas. ASVs are grouped by family, coloured by phyla and sized by mean taxon relative abundance. (b) Differences in the mouse gut microbiota beta diversity associated with exposure to radionuclide contamination. PCoA plot based on the Bray–Curtis dissimilarity in mouse gut microbiota profiles among areas contaminated with radionuclides (CH, FH) or uncontaminated (KL, CL, FL) surrounding either the Chernobyl (*A. flavicollis*, *A. sylvaticus*) or Fukushima (*A. speciosus*, *A. argenteus*) nuclear accident sites. Each point represents a single sample, while shape indicates host species, coloured according to radionuclide contamination “treatments”

## 4 | DISCUSSION

Species identity is thought to dominate over environment in shaping wild rodent gut microbiota (Knowles et al., 2019), but it remains

unknown whether host gut microbiota responses to similar anthropogenic habitat characteristics are species-specific or comparable across host species. To obtain a general assessment of the effects of exposure to environmental contamination on gut microbiota, we

sampled two pairs of mouse species (*Apodemus flavicollis*, *A. sylvaticus*, *A. speciosus*, *A. argenteus*) that occur in sympatry in habitats affected by radionuclides derived from the Chernobyl and Fukushima nuclear accidents. We compared the patterns of microbiota response to radionuclide contamination among these four mouse species, with the general prediction that patterns observed in the bank vole (Lavrinenko, Mappes, et al., 2018) should emerge also in mice exposed to similar environmental contamination. In agreement with our hypotheses, we found that three mouse species, like the bank vole, were characterized by (i) similar alpha diversity, irrespective of host radiation exposure levels, (ii) altered community composition in contaminated areas, and (iii) distinct gut microbiota structure when exposed to radionuclide contamination. Contrary to the pattern observed in the bank vole, however, (iv) selection for a more distinct gut microbiota in these mice does not manifest in a systematic increase in Firmicutes to Bacteroidetes (F:B) ratio. The notable lack of gut microbiota response to radiation in one mouse species from Fukushima (i.e., *A. argenteus*) may be due to host escape from most radiation exposure through its unique tree-dwelling lifestyle. Taken together, our data show both general and species-specific impacts on gut microbiota of murine, and also arvicoline (see Figure S3; and also Lavrinenko, Mappes, et al., 2018), rodents living under chronic radiation exposure.

Human activity typically has negative impacts on the biodiversity of macro- and microorganisms (Carthey et al., 2019; Foley et al., 2005), but the effect of environmental radionuclide contamination on microbial diversity is unclear. A negative association between radiation dose rate and alpha diversity has been observed in soil, bird feather and earthworm microbiota (Czirják et al., 2010; Newbold et al., 2019; Romanovskaia et al., 1998; Ruiz-González et al., 2016), but not in some communities of free-living microbes isolated from areas surrounding the Chernobyl and Fukushima accident sites (Hoyos-Hernandez et al., 2019; Ragon et al., 2011; Theodorakopoulos et al., 2017). We found no negative impact of radionuclide contamination on the diversity of mouse gut microbiota (Figure 3; Figures S4 and S5; see also Figure S3a for bank voles). This result suggests that low-dose ( $\sim 2$  mGy day<sup>-1</sup>) radiation exposure experienced by wildlife within the CEZ and FEZ (Beresford et al., 2020; Kubota et al., 2015) does not reduce host-associated microbial diversity. This is perhaps not surprising as many bacteria can withstand chronic radiation exposure (Ragon et al., 2011; Shuryak, 2019). Moreover, a "rewilding" of the Chernobyl and Fukushima landscapes that followed human abandonment of these sites (Deryabina et al., 2015; Lyons et al., 2020; Perino et al., 2019; Webster et al., 2016) might have widened the niche space available to microbes (Gellie et al., 2017). This in turn could even have concomitant positive effects on the diversity of host-associated microbiota (Hanski et al., 2012). As such, a similar diversity of mice gut microbiota irrespective of radiation exposure levels reinforces patterns found in the bank vole gut microbiota (Lavrinenko, Mappes, et al., 2018). The implication is that exposure to environmental radionuclides does not reduce gut microbiota diversity in wild rodents.

Host diet, habitat niche segregation features and/or host phylogeny can potentially explain differences and similarities in the gut microbiota of two pairs of mouse species (Figure 2; Figure S2; see also Figure S3 for differences between mice and voles) (Perofsky et al., 2019; Youngblut et al., 2019). Despite these effects of host species, our data show a general impact of exposure to radionuclides on structuring the gut microbiota of three out of four species of *Apodemus* mice. The sympatric species pairs share the same environments, and thus should experience comparable radiation exposure. This assumption may well hold true except when there is an apparent "radiation escape" due to some features of host ecology that modify radiation exposure intensity (Beresford et al., 2020; Shuryak, 2020). That the *A. argenteus* gut microbiota was not structured by radionuclide contamination is striking and suggests the microbiota species escape radiation. The most plausible explanation here is that the arboreal lifestyle of *A. argenteus* (Oka, 1992) lessens the average external radiation exposure for this species compared with the sympatric, soil-dwelling *A. speciosus* (Shuryak, 2020; Stark et al., 2017). Given that disruptions of the gut microbiota in early life have been shown to have persistent lifelong effects on microbiota and host performance (Knutie et al., 2017), such radiation escape can be particularly important during early life as *A. argenteus* raises young in tree cavities (Oka, 1992). Indeed, radiation dose rates even at 1 m height can be up to two-fold lower than at ground level (Kubota et al., 2015) because most radionuclides derived from the Fukushima accident were deposited in the topsoil and leaf litter (Hashimoto et al., 2013). There are no estimates of the fraction of time this species spends on trees vs. the ground, yet given that we captured this species using live traps placed on the ground, they at least sometimes descend to the ground when foraging. As both *A. argenteus* and *A. speciosus* forage on contaminated dietary material, they exhibit similar radiation exposures from internal sources (Table 1). However, their internal dose typically accounts for less than 10% of the total absorbed radiation dose (Table 1) (Kubota et al., 2015). Hence, rather than any specific radioresistance (Shuryak, 2020), it is most likely that the tree-dwelling lifestyle of *A. argenteus* helps them to escape most external radiation exposure, resulting in a lack of association between their gut microbiota and soil radionuclide contamination (Figure 4). It is important to note that our estimates of external radiation dose for *A. argenteus* are extrapolated from experimental TLD data on soil-dwelling rodents (see Table S3; and Beresford et al., 2008; Chesser et al., 2000; Lavrinenko et al., 2020). Data from TLDs fitted on *A. argenteus* are required to quantify the effect of species-specific variation in space use on radiation exposure (Beresford et al., 2020; Shuryak, 2020). The finding of an apparent importance of host lifestyle offers an interesting counterpoint for future analyses into the effects of radiation or any other toxic exposure on a host and its associated microbiota.

Numerous studies have sought a reliable biomarker of exposure to radiation (Lourenço et al., 2016; Zhang & Steen, 2018). The Firmicutes to Bacteroidetes (F:B) ratio in the bank vole gut microbiota has a strong positive association with the level of radiation exposure (Lavrinenko, Mappes, et al., 2018). Although the Firmicutes and

Bacteroidetes drive compositional changes in response to radiation exposure also in *Apodemus* mice (Figure 4a), the lack of a systematic increase in F:B ratio in relation to radiation exposure in all species (Figure S10) indicates that the F:B ratio is not a general biomarker of exposure to radiation. This is perhaps not unexpected given the diversity of metabolic functions within the bacterial phylogeny (Lagkouvardos et al., 2019) and the probable species- or strain-specific responses to any changes in host physiology and/or diet that accompany exposure to radionuclides (Kesäniemi et al., 2019; Lavrinienko, Mappes, et al., 2018). Indeed, exposure to radionuclides elicits both an increase and a decrease in the proportion of ASVs assigned to higher taxa, such as the families S24–7, *Ruminococcaceae* and *Lachnospiraceae* (Figure 4a). Using a simple metric of microbiota community composition, such as F:B ratio, to define host condition thus masks underlying heterogeneity in bacterial function (Ridenhour et al., 2017). As such, this finding suggests the overall limited utility of such broad taxonomic microbiota measures as biomarkers in ecotoxicology (Tu et al., 2020).

Gut microbiota can influence host fitness (Alberdi et al., 2016) and it is an important next step to identify whether changes in the gut microbiota of mice exposed to radionuclide contamination impact host health. The bacterial clade that emerges from this study as particularly responsive to radiation exposure, the S24–7 family (other proposed names: Homeothermaceae or Muribaculaceae) (Figure 4a), represents an interesting target for such work. This could pose some challenges, as members of the S24–7 are difficult to culture and only recently have their genomes been characterized (Lagkouvardos et al., 2019; Ormerod et al., 2016). Genome-resolved metagenomic analysis of the S24–7 indicate that it is a speciose (~700 predicted species) and metabolically diverse family, whose members may utilize complex carbohydrates such as starch, hemicellulose and pectin, and also host-derived glycans (Lagkouvardos et al., 2019). Such functional capacity of the S24–7 is consistent with *Apodemus* mice diets and can be beneficial during seasonal fluctuations in resource (seeds) availability (Butet & Delettre, 2011). Metabolic diversity within the S24–7 family probably accounts for the lack of a unified response to radiation (Figure 4a), and similar differential responses of S24–7 to external stimuli have been reported in studies on captive rodents (van Leeuwen et al., 2020; Ridenhour et al., 2017; Smith et al., 2019). Future studies would need to combine manipulative field experiments with metagenomic analyses to quantify gut microbiota functional profiles and link compositional changes to potential changes in bacterial function in the gut microbiota of mice exposed to radiation.

Extensive sampling of multiple host species inhabiting both the Chernobyl and Fukushima accident sites provides a rare opportunity for a synthesis of how radiation exposure impacts rodent gut microbiota. Here we demonstrate that the effects of radiation exposure upon the gut microbiota are generally consistent across host genera (*Apodemus* and *Myodes*; see Figure S3; and also Lavrinienko, Mappes, et al., 2018). Such general effects of radiation could result from (i) indirect impacts through changes in wider environment and/or available diet, or (ii) a direct impact on animal host and/or its microbiota.

Hypothesized indirect impacts such as dietary shift cannot fully explain the patterns observed in this study. Dietary shift is unlikely to occur concurrently in murine and arvicoline rodents that inherently differ in dietary ecology (Butet & Delettre, 2011; Lavrinienko, Mappes, et al., 2018). Similarly, the likelihood that changes in mouse gut microbiota reflect comparable ecological impacts of radiation on generally distinct environments at both the Chernobyl and Fukushima study sites is rather low. Although radiation exposure may act directly as an environmental filter on certain individual taxa, we find no difference in gut microbiota richness between radionuclide contamination treatments in three of the four mouse species studied, and the bank vole (Figure 3; Figures S4 and S5; for voles see Figure S3a; and also Lavrinienko, Mappes, et al., 2018; Lavrinienko, Tukalenko, et al., 2018). Hence, it is conceivable that exposure to radiation is associated with selection targeting either features of host physiology (metabolism, immunity; see Kesäniemi et al., 2019), which in turn affect microbiome assembly or specific gut microbiota profiles that provide the host with essential services. Whether changes in the gut microbiota of bank voles and mice living under chronic exposure to radiation facilitate or impede their animal hosts' overall responses to irradiation under natural conditions remains to be quantified.

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## AUTHOR CONTRIBUTIONS

A.L., P.C.W. and T.M. conceived project design; A.L., E.T., K.K., T.M., Z.B. and T.A.M. conducted field surveys and sample collection for the experiments. A.L. performed laboratory work and completed sequence data analyses, with additional support provided by A.H. and R.H.. A.L. wrote the manuscript with contributions from all other authors. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The raw sequence data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession

no. PRJEB44039 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB44039>). The data sets (QIIME2 files), metadata and code for analyses described in this work are available at: <https://github.com/alavrinienko/hot-mice>, with the final version also archived in Zenodo at: <http://doi.org/10.5281/zenodo.4670304> (Lavrinenko, 2021).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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