Experimental evolution of a mammalian host and associated microbiome: the genetic and maternal effects in bank voles selected for herbivorous capability

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What is already known

Mammalian herbivory represents a complex adaptation requiring evolutionary changes across all levels of biological organization and in addition cooperation with microbiome. Thus, evolution of herbivory is considered as an apparent example of “hologenomic evolution".

What this study adds

However, few attempts have been undertaken to tests assumptions underlying the concept of hologenomic evolution based on the experimental evolution approach. Results of our experiment based on lines of bank voles selected for herbivorous capability showed that selection on the host performance trait leads to genetic changes in the host that promote the maintenance of a beneficial microbiome, which is consistent with the assumptions underlying the concept of hologenomic evolution.

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Experimental evolution of a mammalian holobiont: the genetic and maternal effects in bank voles selected for herbivorous capability

Abstract

Mammalian herbivory represents a complex adaptation requiring evolutionary changes across all levels of biological organization, from molecules to morphology to behavior. Explaining the evolution of such complex traits represents a major challenge in biology, simultaneously muddled and enlightened by a growing awareness of the crucial role of symbiotic associations in shaping organismal adaptations. The concept of "hologenomic evolution" includes the partnered unit of the "holobiont", the host with its microbiome, as a selection unit that may undergo adaptation. Here, we test some of the assumptions underlying the concept of hologenomic evolution using a unique experimental evolution model: lines of the bank vole (*Myodes* = *Clethrionomys glareolus*) selected for increased ability to cope with a low-quality herbivorous diet and unselected control lines. Results from a complex *nature-nurture* design, in which we combined cross-fostering between the selected and control lines with dietary treatment, showed that the "herbivorous" voles harbored a caecal microbiome with altered membership and structure, and changed abundances of several phyla and genera, regardless of the origin of foster mothers. Although the differences were small, they were statistically significant and partially robust to changes in diet and housing conditions. Microbial characteristics also correlated with selection-related traits at the level of individual variation. Thus, the results support the hypothesis that selection on a host performance trait leads to genetic changes in the host that promote the maintenance of a beneficial microbiome. Such a result is consistent with some of the assumptions underlying the concept of hologenomic evolution.

Introduction

One of the main challenges in biology is to explain the evolution of complex adaptations, allowing realization of the astonishing variety of lifestyles. These evolutionary adaptations often require extensive changes across levels of an animal's organization, from molecules to morphology and behavior (Swallow et al. 2009). There is also a growing awareness of the crucial role of symbiotic associations in shaping the evolution of diverse phenotypes and life strategies, from the archaic evolution of the eukaryotic cell to relatively “recent” evolution of mammalian herbivory (Alberdi et al. 2016; Henry et al. 2021). This growing body of knowledge has led to the development of the “hologenome” theory of evolution, a multi-level selection theory arguing that natural selection and evolution can act through a conglomerate biological unit of the “holobiont”, i.e., animal (or plant) hosts along with their associated “microbiome” (or “microbiota”), and hence modify the whole “hologenome” (genes of the host and of the microbiota) (Zilber-Rosenberg and Rosenberg 2008). Although the concept is appealing and supported by results of numerous experimental and comparative studies, e.g. (Sharon et al. 2011; Zepeda Mendoza et al. 2018), its interpretation and usefulness in understanding evolution is subject to debate (Bordenstein and Theis 2015; Moran and Sloan 2015; Queller and Strassmann 2016; Theis et al. 2016; Rosenberg and Zilber-Rosenberg 2018; Rodrigo 2023), and only a few attempts have been undertaken to test its specific assumptions or predictions directly using the experimental evolution approach (Hoang et al. 2016; Kohl et al. 2016). Here we present such a test based on a unique model, lines of a non-laboratory rodent, the bank vole (*Myodes = Clethrionomys glareolus* Schreber 1780), selected for an improved capability to grow on herbivorous diet (Sadowska et al. 2008). Specifically, we asked whether the selection leads to genetic changes in the host that favor the maintenance of an altered microbiome composition that is beneficial from the perspective of the selected trait. If microbiome composition is treated like any other host trait, such a correlated response to selection would be interpreted as evidence of a genetic correlation between the two traits - the selected trait and the "microbiome"; from the perspective of the hologenomic evolution concept, such a correlation would support some of its basic assumptions (Bordenstein and Theis 2015; Theis et al. 2016; Rosenberg and Zilber-Rosenberg 2018).

Herbivory, in particular, is a complex adaptative strategy widely recognized as requiring partnership of hosts and microbes (Mackie 2002; Stevens and Hume 2004; Kohl et al. 2014). Across mammalian species, the taxonomic and functional composition of the gut microbiome is structured by host diet, gut anatomy and phylogenetic history (Ley et al. 2008; Muegge et al. 2011; Brooks et al. 2016; Weinstein et al. 2021). Controlled laboratory experiments, particularly those with germ-free rodents, provided insight into specific effects of particular bacteria and mechanisms of their function (Bäckhed et al. 2004; Heijtz et al. 2011; Laukens et al. 2015). However, several knowledge gaps exist regarding our understanding of hologenomic evolution towards mammalian herbivory: in particular, how the beneficial “host – gut microbiota” associations might coevolve (Hoang et al. 2016; Koskella and Bergelson 2020).

Experimental evolution bridges the gap between comparative and phenotypic-manipulation studies, and offers potential to test hypotheses concerning micro-evolutionary processes and/or mechanisms at various steps of biological organization, from molecular to behavioral (Garland and Rose 2009). This powerful approach has been underutilized in the research on gut microbiota (Hoang et al. 2016), but recently several experiments have shown that applying a selective regime to the host can lead to changes in host microbiome (Kohl et al. 2016; Brown et al. 2019; Wang et al. 2020, 2021; McNamara et al. 2021, 2023; Hanhimäki et al. 2022; Janssens et al. 2022). In a complex experiment, in which lines of high- and low-runner rats derived in classical selection experiment were used to produce inbred strains with matched or mismatched nuclear and mitochondrial genomes, Zhang et al. (Zhang et al. 2020) showed that the differences in host genomes resulted in differential acquisition of the microbiome, which in turn affected several organismal-level physiological and behavioral traits, including those related to the selection trait. However, few other experimental evolution studies have examined the role of the microbiome in determining host performance, or the stability of the alterations with respect to environmental factors.

Given that microbiomes are communities, they are subject to processes of microbial dispersal and ecological community drift (Kohl 2020; Chen et al. 2023). Thus, to show that differences in microbiomes are at least partly determined by the host genes, the potential for microbial exchange within experimental designs should be considered. For example, in a study on rats divergently selected for saccharine preference, which developed distinct microbiomes, microbiome exchange was allowed through cohabition for 7 days (Dess et al. 2020). The differences between the microbiomes were mostly maintained, which suggest stability of the microbial alteration, but a beneficial role of that difference in the selected trait was not demonstrated. Similarly, in another study, in which the gut microbiome of mice from lines selected for submissive or dominant behavior was transplanted into germ-free mice, the recipients developed some of the donors' behavioral and physiological characteristics, but their performance in the selection trial was not significantly altered (Agranyoni et al. 2021). In two experiments on fish selected for mass gain, fish from the selected and control lines hosted different microbiomes regardless of diet (Biasato et al. 2022; Torrecillas et al. 2023), but fish from the distinct lines were not allowed to exchange microbes. Thus, to our knowledge no previous selection experiment has simultaneously documented the stability of differentiated gut microbiomes with respect to dietary variation and microbial exchange, and the beneficial role of the microbial differences with respect to the selected trait.

Here, we employ an ongoing selection experiment on bank voles, comprising four random-bred control (C) lines and four "herbivorous" lines (H) selected for an improved capability of juveniles to grow or maintain body mass during a short 4-day test with low-quality diet (Fig. S1) (Sadowska et al. 2005, 2015; Maiti et al. 2019; Lipowska et al. 2020). In a preliminary study, Kohl et al. (2016) showed that voles from the H lines had a greater diversity and modified composition of the bacterial community inhabiting the caecum and forestomach. These differences were observed in adult individuals fed a standard diet throughout their entire life. However, the parents of these animals had experienced a short episode with the special diet as a part of the selection procedure a few months before mating, and it is known that dietary fiber can alter the retention and transmission of fiber-degrading gut microbes across generations (Sonnenburg et al. 2016; Enav et al. 2022). Therefore, the effects of selection *per se* (genetic differences) could not be firmly distinguished from the effect of diet on the microbiome, carried-over to the offspring through vertical transmission (maternal environment effect). Still, one hypothesis could be that genetically-based host modifications select for a modified bacterial community (Kovacs et al. 2011; Goodrich et al. 2014; Brooks et al. 2016), which in turn increased the host's performance in the selection trial.

Here, we combined experimental evolution with cross fostering and diet manipulation, and used the *nature-nurture* scheme (Fig. 1) to ask a) how both the genetic background and early-life maternal environment affect the bank voles' performance in coping with the herbivorous diet and their gut bacteria composition, b) whether the hypothetical differences in microbiome between the selected and control lines persist under different diets, and c) whether the performance is correlated with the microbiome community characteristics. Newborns were cross-fostered at the birth day either between mothers from the alternative linetypes (the H and C selection line groups) or within the same linetypes. Cross-fostering is widely used in experimental evolution to disentangle the genetically based differences from maternal environmental effects (Cadney et al. 2021), and can also be used to minimize the effects of vertical transmission of bacteria from biological mothers in gut microbiome studies, because cross-fostering effectively shifts the microbiome composition (Daft et al. 2015). At the age of 33 days, animals were subject to 5-day feeding trials on the standard or low-quality diet, during which both body mass balance and digestive efficiency were measured. Finally, samples of caecum contents were taken for molecular analyses of bacterial composition (Fig. 1).

We hypothesized that the selection has resulted in genetic changes in voles that allow for the preferential and stable maintenance of an altered microbiome composition. If true, the microbiome characteristics should differ between the H and C lines 1) regardless of diet and 2) regardless of the linetype origin of their foster mothers. We also hypothesized that the differences in microbiome characteristics have functional effects related to the selected trait. If true, we expected that 3) some of the microbiome characteristics should be correlated with the selection-trial related performance traits at the level of individual variation. Verification of these hypotheses will provide support to some of the assumptions underlying the concept of hologenomic evolution, in particular the assumption that the host genome regulates the microbial composition, which in turn affects the host performance (Zhang et al. 2020; Rodrigo 2023). Finally, if the microbiome composition is also shaped by the microbial transfer from foster mothers, some of the functional benefits should be conferred by cross-fostering. If true, then 4) the selection-related performance traits should also depend on the line-type origin of the foster mother.

Methods

Animal model and the selection experiment

We used bank voles (*Myodes = Clethrionomys glareolus*) from generations 27-28 of an artificial selection experiment comprising four replicate "Herbivorous" (H) lines selected for the ability of juveniles to grow or maintain body mass during a 4-day trial with a low-quality diet (diluted with dried grass powder), and four unselected Control lines (C; Fig. S1). The rationale and protocol of the experiment, and breeding conditions, are presented in previously published papers (Sadowska et al. 2008, 2015; Lipowska et al. 2022) and in the Supplementary Methods. In generation 25, the last in which the selection was performed, voles from the H lines gained 1.55±0.97g in the trial (mean±SD; 7.4% of the initial body mass), whereas those from C lines gained only 0.10±0.89g (0.5% of the initial body mass).

The animals are maintained at constant temperature (20±1°C) and photoperiod (16:8 light:dark), and, except the selection trial, are fed a standard rodent chow: 23.9% protein, 4.5% fat, 5.3% fiber, 14.3 kJ/g metabolizable energy in dry mass; Labofeed H, Kcynia, Poland).

The procedures on animals were approved by the 2nd Local Institutional Animal Care and Use Committee, Institute of Pharmacology PAN in Kraków (decisions 99/2017, 258/2017), in accordance with the EU directive 2010/63/EU. This study is reported in accordance with ARRIVE guidelines.

The cross-fostering procedure

To test the hypothesis that the voles from the H lines host an altered microbiome irrespective of maternal transmission, we performed a cross-fostering experiment. The experiment was performed on second litter offspring of 50 H-line and 50 C-line females from generation 27 (Fig. 1; more details are in Supplementary Methods, available online). The animals from the parental and grandparental generation were not subjected to the selection test. The cross-fostering was performed for whole litters on day of birth, between the C-line and H-line mothers (CH, HC - where the first and the second letter denote the biological and foster mother, respectively), or between mothers representing the same linetype (CC, HH), but not the same replicate line. The procedure was spread across 9 consecutive days. In total, 560 pups were exchanged, and 511 pups in 97 families reached the weaning age (4 to 21 per each combination of the replicate lines). Detailed information about the number of animals in experimental groups at all stages of the experiment is provided in Table S1 (available online). At the age of 17 days, the pups were weaned and moved in groups of up to 5 siblings per cage to individually-ventilated cages (AERO Mouse IVC Green Line: Tecniplast, Italy), which prevented microbiome exchange between the animals from different families.

The feeding trial

At the age of 33 days 479 animals (4-17 pups per replicate line combination) were separated into individual cages and randomly assigned to four combinations of two factors: two categories of diet and two categories of cage type (Fig. 1; details of the procedure and the rationale of using the two cage types are explained in Supplementary Methods, available online). The standard diet (SD) was the same diet as used in the regular maintenance (see above); the low-quality diet (LQD) was similar to that used in the H-line selection tests, but containing less plant material (pellets made of the mixture of 60% Labofeed H and 40% powdered dried grass: 20.4% protein, 4.4% fat, 16.1% fiber, 11.4 kJ/g metabolizable energy in dry mass). The experimental food was less challenging than that used in selection tests because several voles from the unselected C lines might be unable to cope with such food and could die within the 5-day trial. The "standard" cage type (SC) was the same as applied during the H-line selection tests: open-top (model 1264C, Tecniplast, Bugugiatte, Italy), fitted with sawdust bedding. The individually ventilated cages (IVC) were the same as used in the post-weaning period, but fitted with "metabolic cage" type perforated plastic bottoms suspended above the cage floor instead of bedding, which allowed to collect all uneaten food and feces. Water was available *ad libitum*.

The animals were habituated to the cages for three days (with *ad libitum* access to the standard diet), and then the 5-day feeding trial was started (day 0; Fig. 1). The animals were weighed, moved to fresh cages, and given either SD or LQD. In the standard cages, the food was provided in excess to the overhead feeder. In the metabolic cages, a pre-weighed portion of ca 12g food was provided, and weighted samples of the food were taken for measuring dry mass content. At days 1 and 3 the animals were weighed and either returned to the same cage (standard cages group) or moved to fresh cages with a pre-weighed, 23-g portion of food (metabolic cages). Uneaten food and feces collected from the metabolic cages were sorted, dried, and weighed (±0.001g). The rate of food consumption (FC, g/day) was calculated for days 1-5 as the difference between the dry mass of food provided and dry mass remaining in the cage. The rate of effective food digestion (FD g/day; a proxy for metabolizable energy intake) was calculated as a difference between the food consumption and feces production, and apparent digestive efficiency (ADE, %) as the FD/FC ratio.

At day 5 the animals were weighed, euthanized with isoflurane (Aerrane, Baxter, USA) and dissected. The caecum was extracted and its contents were transferred to Eppendorf tube. The tubes were immediately put on dry ice and stored in -80˚C within 2.5 hours.

Animal welfare was monitored daily throughout the experiment. Nineteen individuals were excluded (14 died, 4 showed signs of poor health, 3 were accidently exposed to external microbial sources), and further analyses were performed on 458 animals representing 97 families.

Microbial Sequencing and Analysis

Microbial DNA was extracted with DNeasy Power Soil Pro kit (Qiagen, Germany), according to the producer recommendation. After partial thawing on wet ice, the contents of the tube were mixed with a flame-sterilized spatula, and a subsample of approximately 150mg was taken for the extraction. We used the primers 515F and 806R to target the V4 region of the 16S rRNA gene, a two-step PCR library preparation protocol compatible with the Earth Microbiome Project (Method 4 in: (Glenn et al. 2019; Marquina et al. 2021)). Each sample was indexed, pooled, and sequenced by Novogene (UK) using the Illumina Novaseq PE250 technology. Approximately 50,000 raw read pairs per sample were obtained (more details: Supplementary Methods).

The sequences were processed using the QIIME2 bioinformatic package (Bolyen et al. 2019; Marizzoni et al. 2020; Prodan et al. 2020). We trimmed primers and assembled sequence pairs using the *PEAR* tool (Zhang et al. 2014), with a minimum overlap of 15b, minimum quality threshold of 30, and min-max assembly lengths of 252b-300b. Reads were clustered into amplicon sequence variants (ASVs) with the *deblur denoise-16S* tool, aligned, and used to construct phylogenetic trees using the *phylogeny align-to-tree-mafft-fasttree* function. The taxonomic information of ASVs was obtained with the feature-classifier *clarify-consensus-vsearch* tool and the *SILVA 138* database (Quast et al. 2013). The sequences derived from mitochondria, chloroplasts, archaea, and singleton sequences were excluded from the list with the *feature-table filter-features* function. The feature table was rarefied to 10,227 sequences per sample with the *feature-table rarefy* function. Twenty of such rarefied tables were generated for further bootstrap analyses. One individual with only 7808 reads returned was excluded from analyses based on rarefied results, but was included in estimates of the bacterial mean abundance and analyses of the morpho-physiological traits.

The *diversity alpha* tool within QIIME2 was used on each of the rarefied tables to obtain three alpha-diversity metrics: number of observed ASVs (N_{ASV}), Shannon diversity index and Pielou evenness index. Weighted and unweighted UniFrac distance matrices were obtained for each of the rarefied tables with the *diversity beta-phylogenetic* too, and a principal coordinate analysis (PCoA) plot was generated using the *diversity pcoa* function. Both the alpha-diversity metrics and the UniFrac matrices were averaged across the twenty repetitions to obtain the bootstrapped estimates.

Based on these initial results, we noticed 39 animals (8.5%) with a strikingly low microbiome diversity, and forming a separate cluster both in the heatmap and the beta-diversity plots (Supplementary Results Fig. S2, Table S3-S4, available online). Those individuals were distributed nearly evenly across all the experimental groups, and could be nearly perfectly distinguished by a single criterion – the presence of bacteria from *[Clostridium] innocuum* group. Those voles had also a lower body mass and lower food digestibility (Supplementary Results, available online). Therefore, as those outlying individuals would distort the analyses of both the microbial and the physiological traits, we removed them from further investigation, leaving 419 individuals for the proper statistical analyses.

Statistical analyses

The statistical analyses included three main parts (details in Supplementary Methods, available online). First, to test the effects of the experimental factors on body mass, body mass change during the feeding trial (MD_{FT}; g/5 days), rates of food consumption and effective digestion (FC, FD; g/day), apparent digestive efficiency (ADE; %), and the three alpha diversity metrics, cross-nested mixed ANCOVA models were fitted with SAS Mixed procedure (SAS v. 9.4 (SAS Institute Inc. 2011)). All the models included the selection direction (linetype), origin of both the biological and foster mother (H *vs* C lines), diet (SD *vs* LQD) and sex as the main fixed factors, interactions between these main factors, and respective random effects. This basic model structure was further expanded to accommodate additional factors and covariates (body mass, day and time of the measurements, and litter size at weaning) adequate for specific analyses. Except for FC, FD and ADE, which were measured only in IVC cages, analyses of the other traits were performed both separately for each of the cage types, and for all individuals (with the cage type as cofactor). Initial models included interactions between the main factors (and respective random interaction term), and were step-wise reduced by removing non-significant interactions. However, interactions between the three focal factors, the origin of biological and foster mother and diet, were always retained in the final models. Two individuals were excluded as severe outliers (absolute value of studentized residual ≥ 4.0) from analyses of MD_{FT}, and two from analyses of ADE (and also FC and FD).

To analyze the effects of the focal factors on the multivariate beta-diversity characteristic of the microbial community we used permutational multivariate analysis of variance (PERMANOVA, with 9999 permutations) implemented in *adonis2* function of QIIME2 and R (v4.3.0) *vegan* package (v2.6-4) (Anderson 2017; Oksanen et al. 2022). The analyses were performed for both the unweighted

UniFrac distance matrix (describing the community membership) and the weighted UniFrac distance matrix (describing the community structure). The models included the same cofactors, covariates and interactions as the univariate models described above, and were similarly step-wise reduced. As the analyses showed significant interactions between the focal factors, the analyses were performed also separately for the diet and mother-origin subgroups. Although *adonis2* PERMANOVA can handle random effects (Anderson 2017; Oksanen et al. 2022), it cannot cope with unbalanced nested designs. Therefore, in these analyses the random effects of replicate lines were not included (c.f. (McNamara et al. 2021; Hanhimäki et al. 2022)).

To gain insight in what taxonomic groups contributed to the differences in the microbiome communities between the experimental groups, we performed univariate analyses of abundances of 11 phyla and 111 genera (omitting the phylum Fusobacteriota, present largely in the 39 individuals hosting bacteria from [*Clostridium*] *innocuum* group and any genera present in <10% of individuals). To avoid an excessive number of tests and problems with non-independence of tests performed at different taxonomic levels, we limited the analyses to phyla, which provides a broad perspective, and to genera, where we can expect the information about the abundance of a particular taxon to be associated with a particular function. In these analyses unclassified and uncultured bacteria from higher taxa were operationally treated as “genera”. The analyses were performed for relative abundances with *adonis2* function, and for the bias-corrected “absolute” abundances, in which log fold-changes of the abundances are analyzed (*ancombc2* function in R package ANCOMBC, v. 2.4.0; (Lin and Peddada 2020a, 2020b)). Details and rationale of applying the two approaches are explained in Supplementary Methods. P-values obtained in these analyses were corrected using the Benjamini-Hochberg False Discovery Rate BH-FDR) correction for multiple comparisons (Benjamini and Hochberg 1995).

Finally, we tested correlations between performance traits measured during the feeding trials (MD_{FT}, FC, FD, ADE) and microbial characteristics at the level of individual variation, testing partial correlations within main factor groups. To assess the association with the overall microbial community membership and structure, we applied the same *adonis2* PERMANOVA models as described above, but with the performance traits and their interaction with diet as additional predictors. Similarly, we used *ancombc2* to analyze the association with log-fold differences in "absolute" abundances of particular taxa (11 phyla and 111 genera). The correlations with relative abundances analyzed with linear models (R *lm* function), with the performance traits as the dependent variable, and the microbiome traits as predictors (and the same set of the fixed predictors as used in analyses aimed at testing the effects of experimental factors on the performance traits). In both of the analyses of correlations with abundances, P-values were BH-FDR corrected.

In all analyses we assumed conventional $p<0.05$ as the threshold of significance.

Results

Dominant microbiome taxa and alpha diversity

In the 458 caecal samples, 5058 amplicon sequence variants (ASVs) were identified, which were classified into 12 phyla, and 70 taxonomic families (Table S2, S3, available online). Majority of ASVs (4498; 88.9%) were identifiable to 147 genera (122 with confirmed taxonomy, Table S2, S3, available online). As we already mentioned in Methods, 39 voles with a strikingly distinct bacterial community (Fig. S2, available online) were excluded from the main analyses.

The three alpha diversity metrics, the number of ASVs (N_{ASV}), Shannon index diversity, and Pielou index of evenness, were higher in animals fed the LQD diet than those fed the SD diet (all $p<0.0001$), and higher in females than in males (N_{ASV}: $p=0.027$, Shannon index: $p=0.011$; Pielou index: $p=0.039$; Fig. 2; Table S5, S6, available online). The linetype origin of the biological or foster mothers, or interactions involving these factors, were not significantly associated with these indices ($p>0.13$), with a possible exception that Pielou index tended to be higher in animals reared by foster mothers from H than C lines ($p=0.064$).

The microbiome beta-diversity and abundance of particular taxa

Diet was the major factor affecting the microbiome, explaining 8.7% of the joint variation in the community membership (unweighted UniFrac distances), and 33.4% variation in the community structure (weighted UniFrac; PERMANOVA, both $p<0.001$; Table S7, available online), with the effect of diet clearly seen on the first two PCoA axes (Fig. 3A,C). The community characteristics did not differ significantly between sexes, but differed between the cage types (about 0.5% of the total variance, $p\leq0.007$).

Selection linetype of both the biological and foster mothers significantly affected the community membership (PERMANOVA on unweighted UniFrac distances; $p<0.001$) and structure (PERMANOVA on weighted UniFrac distances; origin of the mother: biological: $p=0.003$; foster: $p=0.006$; Fig. 3; Table S7, available online). Distinctions based on these variables are visible only weakly on the plot of the first two PCoA axes of unweighted UniFrac distances (Fig. 3A) and more clearly only on further PCoA axes (Fig. 3B,D), as they explained only a small proportion of total variance: about 1% for the community membership and 0.6% for the community structure. In addition, the interaction between the biological and foster mother linetypes was significant ($p\leq0.003$), but, importantly, the differences due to the mothers' linetype appeared relatively consistent across the diet types, in that they were only weakly affected by interactions with diet type (community membership – diet × biological mother: $p=0.031$; community structure – diet × biological × foster mother: $p=0.025$; other interactions not significant; Table S7, available online). Separate analyses for diet groups, and finally for both diet and mother types, confirmed that both the biological and foster mother origin significantly and independently affected the community membership and structure (Table S7 available online).

We analyzed abundances of 11 phyla and 111 genera with univariate models using two metrics: untransformed relative abundances, and bias-corrected log fold-changes of absolute abundances (using *ancombc2*; Fig. 4; Tables S4 S8-S11, available online). Diet type significantly affected the relative and absolute abundances of numerous phyla and genera. Both methods showed that the LQD significantly increased the abundances of Desulfobacterota, Actinobacteriota and Patescibacteria, and decreased in Spirochaetota, Proteobacteria and Verrucomicrobiota. The abundances of several taxa appeared significantly associated with sex, body mass, litter size, time of sampling, or differed between cage types (Tables S8-S11, available online).

Irrespective of these confounding factors, biological selection linetype significantly affected the relative and absolute abundances of several taxa (Tables S4, S8-S11, available online). The effect of linetype was usually larger in animals fed SD than those fed LQD (and interactions with diet were often significant). Voles from the H lines fed SD, irrespective of the origin of the foster mother, had a significantly higher relative abundances of Proteobacteria (C: 2.73%, H: 3.63%, p=0.004) and Actinobacteriota (C: 0.32%, H: 0.68%, p=0.003), and relative abundance of several genera (e.g., Firmicutes: *Lactobacillus* – C: 6.59%, H: 8.83%, p=0.005; *Ileibacterium* – C: 0.71%, H: 2.09%, p=0.02; Bacteroidota: *Alistipes* – C: 2.45%, H: 2.95%, p=0.046; Verrucomicrobiota: unclassified Puniceicoccaceae – C: 1.00%, H: 1.46%, p=0.020). In some genera the trends were similar in both diet types, and the difference between H and C lines was significant only for both diet groups combined (e.g., Firmicutes: *Ruminococcus* – C: 0.61%, H: 0.90%; p=0.032; Bacteroidota: Rs-E47_termite_group – C: 2.50%, H: 3.30%; p=0.032). For some of the genera the differences were confirmed in analyses of the absolute abundances with *ancombc2* (e.g., *Ileibacterium*, *Ruminococcus*; Table S11, available online). The relative abundances of four genera were significantly lower in voles from H lines, but in the most abundant, an uncultured genus of Christensenellaceae, only in voles fed SD (C: 5.48%, H: 3.46%, p=0.046).

Foster mother selection history, independently of the origin the biological mother, affected relative abundances of 11 genera, and in additional 9 the effect appeared in only one of the diet groups (mostly poorly characterized genera; Tables S4, S9, available online). Importantly, all of the genera identified here were different from those dependent on biological mother origin, with the exception of the unclassified Puniceicoccaceae, for which the direction of the effects of biological and foster mothers was opposite. Voles reared by H-line foster mothers, irrespective of the biological origin, had higher relative abundance of 9 genera (e.g., Firmicutes, both diets: *Lachnospira* – C: 0.06%, H:

0.10%, p=0.034; SD only: *Oscillibacter* – C: 0.38%, H: 0.51%, p=0.017; LQD only: NK4A214 group – C: 0.25%, H: 0.33%, p=0.014; uncultured Ruminococcaceae – C: 2.41%, H: 3.06%, p=0.016; uncultured Peptococcaceae – C: 0.12%, H: 0.16%, p=0.015; Verrucomicrobiota, both diets: uncultured Puniceicoccaceae – C: 0.00%, H: 0.27%, p=0.002), and lower relative abundances of 11 genera, mostly lacking specific genus names (e.g., Firmicutes: Lachnospiraceae A2 – C: 0.68%, H: 0.39%, p=0.002; *Fournierella* – C: 0.36%, H: 0.20%, p=0.002; Campilobacterota: *Helicobacter* – C: 4.61%, H: 3.57%, p=0.011). Foster mother origin affected absolute abundances (assessed with *ancombc2*) of five genera: the abundances of *Fournierella*, Clostridia UCG-014 (Firmicutes), and *Bauldia* (Proteobacteria) were lower in voles reared by H-line foster mothers, and abundances of *Monoglobus* (only on LQD) and unclassified Erysipelotrichaceae (only on SD) (Firmicutes) were higher in voles reared by H-line foster mothers (Table S11, available online).

Abundances of several taxa showed dependencies on interacting factors (Tables S8-S11, available online). For example, *Helicobacter* was influenced by a significant interaction between biological and foster mother, with higher abundance in voles from within-selection transfers (C-C or H-H) compared to voles fostered by a mother from the opposite selection linetype. The abundance of *Monoglobus* (Firmicutes) was higher in voles reared by H-line foster mothers, but only in the LQD group. Several other taxa only exhibited variable differences based on linetype depending on the diet treatment (significant interactions; Tables S8-S11, available online).

Performance in the feeding trial

The body mass (adjusted for cage type and time of day), was larger in males (LSM±95%CI; 23.2±1.2g) than in females (20.7±1.2g; p<0.001), larger in H lines (23.3±1.6g) than in C lines (20.6±1.6g, p=0.028), but did not differ significantly between voles reared by the H- or C-line foster mothers (p=0.19; Fig. 5A; Tables S5, S6, Fig. S3, available online). The body mass at the beginning of the feeding trial (after habituation) was lower in animals moved to the standard cages (SC: 21.6±1.2g) than those kept in the individual ventilated cages (IVC, 22.2±1.2, p=0.051), and the difference was smaller in animals reared by H-line than C-line foster mothers (interaction: p=0.020).

Animals born to H-selected voles exhibiting higher defense of body mass during the feeding trial. However, the magnitude of the effect of selection and associated data (such as digestibility) depended on cage type, so we present statistics separately (Fig. 5B,C; Tables S5, S6, Fig. S3, available online). In the standard cages, as expected, voles fed the low-quality diet (LQD) lost on average 3.18±0.81g, whereas those fed the standard diet (SD) gained 0.37±0.80g (diet: $p<0.001$; Fig. 5B). Voles from the H lines performed better, losing only 0.62 ± 0.81g, whereas those from C lines lost 2.18 ± 0.81g (linetype: $p=0.004$; linetype × diet interaction: $p=0.37$). Results from IVC cages were similar, but with markedly higher mean values of MD_{FT} (Fig. 5C): voles fed the LQD gained on average 0.30±0.57g and those fed SD gained 1.56±0.57g (diet: $p<0.001$). Voles from the H lines gained more mass (1.58±0.64g) than those from C lines (0.28±0.64g, linetype: $p=0.003$), and the difference between linetypes was 0.85g larger in animals fed the LQD (linetype × diet interaction: $p=0.052$). Males gained on average more mass (1.12±0.55g) than females (0.73±0.55g; $p=0.006$), and the difference between the SD and LQD group was larger in males than in females (interaction: $p=0.032$). Foster mother linetype had no effect on MD_{FT} in any of the cage types ($p>0.5$).

The rate of food consumption (FC, g/day), its digestibility (apparent digestive efficiency, ADE, %), and the effective food digestion (FD, g/day, a proxy for metabolizable nutrients and energy intake), were estimated only for the animals kept in the metabolic cages. As expected, FC increased with body mass and animals fed the LQD consumed more food (mass-adjusted FC: 6.02±0.37g/d) than those fed the SD (5.36±0.37g/d; $p=0.002$; Fig. 5D; Tables S5, S6, Fig S4, available online). Voles from H lines consumed more food than those from C lines (H: 5.96±0.39g/d; C: 5.41±0.40g/d; $p=0.019$), and the differences between linetypes were more profound in the LQD (0.81g/d) than in the SD group (0.28g/d; interaction: $p=0.085$). The foster mother linetype or sex had no effect on the adjusted FC.

The apparent digestibility (ADE) of LQD (57.8±1.7%) was much lower than that of SD (78.8±1.7%; $p<0.001$; Fig. 5E, Tables S5, S6, available online). The digestibility decreased with initial body mass of the voles (Fig. S4, available online), but the origin of the biological or the foster mothers or sex had no effect on ADE. The rate of effective food digestion (FD) increased with body mass (Table S5, Fig. S4, available online). Despite a higher FC, the mass-adjusted FD was lower in animals fed the LQD

(3.44±0.22 g/d) than those fed the SD (4.21±0.22; $p<0.001$; Fig. 5F; Tables S5, S6, available online). H-line voles effectively digested more food than the C-line ones (H: 4.00±0.23g/d; C: 3.65±0.24g/d; $p=0.012$), irrespective of the food type (interaction: $p=0.4$).

The correlation between microbial characteristics and performance in the feeding trials

The analyses of partial correlations showed that the four feeding-trial performance traits (MD_{FT}, FC, ADE, and FD), adjusted for the same fixed factors as in the main analyses (presented above), were not correlated with the Shannon index or Pielou index (Table S12, available online). However, digestive efficiency (ADE) was positively correlated with the number of amplicon sequence variants (N_{ASV}; $p<0.001$; Fig. 6A), and the effect was more profound in animals fed the standard diet (interaction: $p=0.012$). On the other hand, food consumption rate was negatively corelated with N_{ASV} ($p=0.005$; Fig. 6B), and therefore the rate of food digestion did not depend on N_{ASV} (Table S12, available online).

Multivariate analyses revealed several correlations between the performance traits and the bacterial community membership (unweighted UniFrac distances) and community structure (weighted UniFrac distances, Table 1).

In a model for all groups combined, both MD_{FT} and its interaction with diet were significantly correlated with the community membership (main effect $p=0.044$, interaction $p=0.047$) and community structure (main effect $p=0.010$, interaction $p=0.037$). In analyses split by diet group, the effect of MD_{FT} was not significant for the community membership ($p\geq0.16$), and marginally insignificant for the community structure (SD diet: $p=0.079$, LQD diet: $p=0.063$). The analyses performed separately for voles from the standard cages showed no correlation between MD_{FT} and community membership ($p>0.15$), but did exhibit a significant correlation with the community structure (both diet types combined $p=0.023$; LQD group $p=0.011$, SD group $p=0.18$). On the other hand, in voles kept in IVC cages, there was a weak correlation with the community membership only in SD group ($p=0.06$), and no correlation with the community structure ($p>0.27$).

The community membership was clearly correlated with FC ($p=0.004$), ADE ($p<0.001$), and FD ($p=0.077$), but when analyses were performed separately for diet groups, relationships between microbiome membership and FC or FD were significant only in voles fed the SD diet (FC: $p<0.001$, FD: $p=0.042$). The community structure was correlated with FC and AD in voles fed the SD diet (FC: $p=0.049$, ADE: $p=0.003$), but not in the LQD diet group, and it was not correlated with FD for any of the groups.

Univariate analyses or partial correlations showed that body mass change during the feeding trial (MD_{FT}) in voles kept in standard cages was correlated with the relative abundance of five phyla, but not with any genus (Table S12, available online). MD_{FT} was higher in voles with greater abundances of the phylum Desulfobacterota ($p=0.022$), Verrucomicrobiota ($p=0.001$), and Actinobacteriota ($p=0.001$; Fig 6E) or with lower abundances of Bacteroidota ($p=0.004$), and tended to increase correlationally with the relative abundance of genus *Lactobacillus* (Firmicutes; $p=0.09$; Fig. 6F). In Verrucomicrobiota and Actinobacteriota, the positive correlations were more profound in voles fed the standard diet (interaction: $p\leq0.005$). In Proteobacteria the positive correlation was present only in voles fed the LQD diet (main effect $p=0.013$, interaction: $p=0.004$). Similar analyses of the bias-corrected absolute abundances using *ancombc2* consistently revealed a positive correlation with MD_{FT} at the phylum level only for Verrucomicrobiota ($p=0.017$), and unique identification of significant correlations for 14 genera (including *Lactobacillus*, *Rikenella*; *Ileibacterium*, *Syntrophomonas*; *Bauldia*; and *Bifidobacterium*; all $p<0.05$; others Table S13, available online).

In voles maintained in the IVC metabolic cages (in which food balance was measured) MD_{FT} was correlated with relative abundance of only one taxon, *Lactobacillus*: voles with a higher relative abundance grew faster ($p=0.002$), irrespectively of the diet type (Fig. 6F, Table S12, available online). Significant correlations with the absolute abundance were present only for a few genera, including *Bauldia* (all $p\leq0.014$; other results: Table S13, available online). Also, MD_{FT} tended to increase with the absolute abundance of *Lactobacillus* ($p=0.09$).

The rate of food consumption (FC) was correlated with the relative abundance of 11 genera (including positive correlations with *Coprococcus*; and negative correlations with: *Oribacterium*, *Ruminococcus*, Clostridia UCG-014, *Ileibacterium*, and *Treponema* (all p<0.05; Fig. 6C; Table S12, available online). FC was clearly correlated with absolute abundance of only *Bauldia* (positive correlation, p=0.013), and in a few the trends were diet-dependent (Table S13, available online).

We also identified taxa correlated with the apparent digestive efficiency (ADE) – three phyla exhibiting negative correlations: Cyanobacteria (p=0.008), Campilobacterota (p=0.073), Proteobacteria (p=0.073) and 17 genera (including positive correlations: *Ruminococcus*, Clostridia UCG-014, *Ileibacterium, Treponema*; *Enterorhabdus*; negative correlations: *Anaeroplasma*; *Rikenella*; *Gastranaerophilales*; direction diet-dependent: *Syntrophomonas*; all p<0.05; Fig. 6D, others Table S12, available online). However, ADE was not significantly correlated with the absolute abundance of any taxon (Table S13, available online).

For several taxa the direction of correlations of the relative abundance was opposite for FC and ADE (Fig. 6). Thus, it is not surprising that few taxa showed correlations with the rate of food digestion (FD), i.e., with the product of the two traits (Table S12, available online). FD was positively correlated with the relative abundance of Lachnospiraceae FCS020 group (Firmicutes), but only in voles fed the standard diet (main effect p=0.027, interaction p=0.027), and tended to be positively correlated with relative abundances of *Coprococcus* (p=0.057) and *Lactobacillus* (p=0.065). FD was not correlated with absolute abundance of any taxon (Table S13, available online).

Discussion

The results of our experiment showed that selection-related traits differed between the selected (H) and control (C) lines of bank voles, with foster mothers having little effect on these performance traits. We also present evidence for small but significant modifications in caecal bacterial community composition due not only to the origin of the foster mother (maternal effects, vertical transfer), but also to the origin of the biological mother (genetic effect of selection *per se*). Importantly, although diet had a profound effect on microbiome composition, selection-related changes were partially robust to dietary change. Furthermore, some bacterial traits were correlated with voles' performance in the selection-related traits. Thus, results from the experimental evolution model system provided support for some of the assumptions underlying the concept of hologenomic evolution, in particular that selection on a host trait leads to genetic changes in the host that promote the maintenance of a beneficial microbiome.

As expected, the vole gut microbiome was most strongly modulated by diet. The bacterial communities of animals fed the grass-diluted, fiber-reach diet, were more diverse than that of voles fed the standard diet, as shown by increased values of all three alpha-diversity traits we analyzed, altered the community membership and structure, and the abundances of most of the bacterial taxa. In general, many of these diet-induced changes reflect previous observations regarding feeding on fibrous diets (Reese and Dunn 2018). While these findings are useful for their confirmatory nature, our main interests for the purposes of this study concern the metrics and taxa which exhibit relationships with selection, cross-fostering, or performance traits.

Voles from the H lines, selected for improved coping with the herbivorous diet, had an altered membership and structure of caecal bacterial community, compared to voles from the unselected control (C) lines, matching the previous report from earlier generations of the same selection experiment (Kohl et al. 2016). The current results present a stronger test of the effects of selection. The effect of the biological selection linetype was repeatable and demonstrated within the context of animals fed either the low-quality or the standard diet, and in animals maintained under different housing conditions. Additionally, the differences are considered robust given their presence after two generations of relaxed selection, in animals whose parents and grandparents had no experience with the low-quality diet. Moreover, we observed these differences in the context of cross-fostering, by which microbial transmission from the opposite selection lines were possible in early life. While a variety of potential mechanisms may underlie these results, which we discuss below, their repeatability suggests that our bank vole system is promising for the interrogating some of the assumptions and mechanisms underlying the process of hologenomic evolution.

Our experimental evolution model was designed to mimic early stages of evolution of herbivorous strategy in mammals (Sadowska et al. 2008), a transition widely regarded as requiring the evolution of the ability to host specific bacterial symbionts. Such an idea could have been implemented in several ways. Perhaps the most apparent target of selection is ability to digest fiber. In consuming plants, herbivores must cope with recalcitrant fiber in their diets, which they often digest through their partnership with microbial symbionts. However, from an organismal and evolutionary perspective, coping with a particular diet in terms of percent digested may be less important than ability of converting food into body growth or offspring. Therefore, we argue that the ability of juveniles to grow or maintain body mass during a period of feeding on the low-quality diet (LQD) is an appropriate proxy for measuring "adaptation" to the herbivorous strategy, and is in agreement with the intended evolutionary scenario under which animals of a non-strict herbivore species may be faced with a temporal shortage of typical food, and selection would favor those individuals that can instantly cope with the herbivorous diet (Sadowska et al. 2008, 2015). Importantly, the experimental evolution approach can reveal the coordinated and multi-level nature of the phenotypic changes. For example, voles from the H lines tended to have a decreased basal metabolic rate, locomotor activity, and hormonal recovery after an acute stress (Sadowska et al. 2015; Maiti et al. 2019; Lipowska et al. 2020), but increased fat content (unpublished data). Including the microbiome as another level at which our selection may have acted offers a more biologically realistic understanding of adaptation towards herbivory.

As such, we observed differences in some traits relevant to herbivory between H and C lines, and these traits were not influenced by cross-fostering (we discuss this aspect later). Voles from the H lines were larger at the beginning of the feeding trial and grew faster during this period on both diets (Fig. 5). The results also showed that voles from the H lines had a higher rate of digestion of the LQD and thus had an increased metabolizable energy intake. This difference was due to an increased rate of food consumption rather than increased digestive efficiency (Fig. 5). The ability to consume and process the low-quality food at a higher rate without compromising digestive efficiency indicates an improved capacity for herbivory in this group, given that there is typically a tradeoff between digestion rate and digestive efficiency (i.e. rate maximizing versus yield maximizing (Karasov and Martínez del Rio 2008)). Such results could be due to increased alimentary size or performance, or improved efficiency of symbiotic digestion at the biochemical level. Additionally, the differences in body mass between groups may contribute, as greater size is generally considered to be an adaptation to the herbivorous strategy to allow for greater food retention and lower relative energy requirements (Demment and Van Soest 1985), though greater body size also presents physiological challenges in the need to absorb and distribute nutrients through the body (Clauss and Hummel 2005).

In addition to the widely known role of gut symbionts in cellulose digestion, there may be other mechanisms by which the gut microbiome facilitates mammalian herbivory (Dearing and Kohl 2017). The gut microbiome is tightly integrated with maintenance of host mass balance, especially through interactions with metabolic physiology (Chevalier et al. 2015; Sommer et al. 2016; Regan et al. 2022). These metabolic interactions might also occur through general interactions with body size, as aspects of gut microbiome are correlated with body size across species (Godon et al. 2016; Reese and Dunn 2018; Sherrill-Mix et al. 2018). Additionally, through the gut-brain axis, the gut microbiome can modulate aspects of feeding behavior and feeding rates (Bo et al. 2020; Shu et al. 2021; Trevelline and Kohl 2022). Thus, the microbial contributions to mammalian herbivory may extend beyond digestion of cellulose, to include other aspects of the animals' energetics, physiology, and behavior that contribute to improved efficiency in converting consumed food to body growth.

We observed significant differences in microbiome structure based on maternal selection line (H versus C), that were independent of cross-fostering, indicating a genetic effect of selection on the host in structuring the gut microbiome. Though we did not recapitulate previously observed differences in alpha diversity between H and C lines, the multivariate analyses revealed a significant effect of the selection direction on the community membership and structure. The effect of selection explained about 1% of the entire variance in these community characteristics. It could be argued that such a small effect has little biological significance. However, this effect concerns the difference between four independent replicate lines of both the selected and unselected control lines, and we have shown that the effect of selection is to some extent robust to disturbances such as exchange of bacteria through cross-fostering and altered diet or housing conditions. Moreover, this effect appeared after only 23 generations of effective selection (Supplementary Methods, available online), i.e., on a very short evolutionary time scale. Thus, in line with other studies based on rodent selection experiments that have reported correlated changes in microbiome composition of comparable magnitude after more generations and with larger differences in the directly selected trait (McNamara et al. 2021, 2023), we believe that the small difference is still biologically meaningful. Focused studies have demonstrated host genetic effects on the microbiome can be imparted through differences in loci related to digestive enzymes, mucins of the gut lining, or adaptive or innate immunity (Goodrich et al. 2014). Though, resolving the relative contributions of genetic and environmental determinants of the microbiome remains a pressing question in the field (Grieneisen et al. 2023). We have reduced the environmental (dietary) and epigenetic effects through studying the microbiome after two generations of relaxed selection, i.e., during which animals had no contact with the special diet. We cannot exclude the possibility of some microbes being transmitted during the birth event (Hansen et al. 2014; Morais et al. 2020). However, for a coordinated experiment to properly match Caesarean-birthed pups, we would have been left an insufficient number of time-paired, surgery-free, recently-birthed mothers to raise the required sample size of pups for our tests. Moreover, although cross-fostering does not eliminate the possibility of transmitting bacteria from biological mothers during the birth or immediately after, such a transmission does not determine the gut microbiota composition (Queller and Strassmann 2016), and it has been shown that cross-fostering effectively shifts the microbiome composition (Daft et al. 2015). Thus, we believe that the combination of relaxed selection with cross-fostering provided a strong basis for assuming that the significant effect of the biological mother linetype origin reflects the host genetic contribution to shaping the gut microbiome composition.

Notably, we observed a significant correlation between microbial species richness and digestive efficiency. Relationships between diversity and function are enigmatic to ecologists and evolutionary biologists, though complex to interpret given the many measures of diversity and of function (Shade 2017; Reese and Dunn 2018). These data suggest a more diverse microbiome to facilitate more efficient digestion. In the context of herbivory, it is typically thought that a greater taxonomic diversity yields higher functional diversity, which is beneficial towards degrading the complex fibers present in plant material (Reese and Dunn 2018). Here, we also observed correlations between bacterial community characteristics and herbivory-related performance traits (body mass balance, food consumption, and digestive efficiency), supporting the notion of functional links between the microbiome composition and whole animal performance.

Our studies suggest several taxa that may play a role in hologenomic evolution towards herbivory in our system. The genus *Ruminococcus*, which is well known to play a role in fiber degradation (Christopherson et al. 2014), was more abundant in voles from H-selected lines, irrespective of foster mother, and also showed a positive relationship with digestive efficiency. Another genus, *Lactobacillus*, which was also higher in H lines, is a dominant genus in the foregut chambers of several herbivorous rodents (Kohl and Dearing 2012; Kohl et al. 2014; Shinohara et al. 2016). Although, counterintuitively, its relative abundance decreased in response to short-term LQD diet, it was positively correlated with the rate of food digestion, a proxy of metabolizable energy intake, and with the selected trait - body mass defense or growth. This is in line with the observation that *Lactobacillus* is associated with growth promotion in malnourished mice through interactions with hepatic growth hormone signaling (Schwarzer et al. 2016). Thus, it may play a role in evolution of adaptation to a low-quality diet not through digestion, but through regulation of metabolism.

Our cross-fostering treatment showed that the linetype origin of the foster mothers also affected the microbiome membership and structure, to about the same extent as that of the biological mothers (~1% of total variation). Thus, some microbes might be acquired through maternal transmission and maintained irrespective of the voles' genotype, or the bacteria abundance was influenced by other maternal environment effects. However, none of the selection-related performance traits was affected by the origin of the foster mother. Thus, the hypothesis that microbiome transfer from the selected H-line voles should provide benefit to those from the unselected C lines has been falsified. Taken at face value, such a result could be taken as evidence that the alteration of the microbiome in the selected H lines did not play a significant role in the evolution of the increased ability to cope with the low-quality diet, and thus undermine the assumption that the selection experiment could be treated as a model of hologenomic evolution. Importantly, however, the taxa associated with foster treatment were unique from those associated with genetic selection direction. For example, voles raised by mothers from the H line, regardless of their biological mother, harbored lower abundances of *Fournierella*, a recently-characterized anaerobic genus first isolated from the human gut (Togo et al. 2017), but abundance of the bacterium did not differ between voles from H and C lines. Conversely, origin of the foster mothers did not significantly affect abundances of those taxa that differed between H and C lines or those that were correlated with the selection-related traits (see the previous paragraph). Such a differential transmission may be due to altered dispersal between foster mothers and pups, including differential transmission of anaerobic and aerobic microbes (Moeller et al. 2018), or differential maternal effects, such as the nutritional or immunological composition of milk (Gopalakrishna and Hand 2020; Keady et al. 2023). At any rate, the results show that the lack of the effect of cross-fostering on the selection-related performance traits does not undermine the claim that the altered microbiome, apparently determined by the host genetic background, plays a positive role in evolution of the improved coping with herbivorous diet in the selected H lines.

To summarize, our results support the hypothesis that selection on a host performance trait leads to genetic changes in the host that promote the maintenance of a beneficial microbiome. Such an outcome is consistent with some of the assumptions underlying the concept of hologenomic evolution, in particularly with the assumption that host genome regulates the microbial composition, which in turn affects the host performance (Zhang et al. 2020; Rodrigo 2023). However, we recognize that our experiment had limitations, one of which was the that it focused only on bacteria. Further research should include other components of the holobiont microorganisms, such as fungi and viruses. Next steps should involve assessing mechanisms underlying the host-microbiome association (what genetic changes in the hosts might confer the robust H-selected microbiome?). Additionally, our results contain a number of interactions and dependencies on other variables. Addressing context dependency is a challenge for the greater fields of ecology and evolution, and the first step is to identify true context dependency versus stochastic or experimental noise (Catford et al. 2022). The necessary patterns to address context dependency will only emerge through studies of hologenomic evolution across systems and experimental designs. We believe continued work with our bank vole system will be a powerful tool in growing understanding the hologenomic evolution of mammalian herbivory, and encourage the development of other similar experimental evolution approaches.

Data and code accessibility statement

Data used in the study and software code applied in bioinformatic and statistical analyses are provided as supplementary material (available on line), and will be published in an open repository upon the paper acceptance.

Supplementary online materials (electronic appendixes)

- a pdf file with supplementary Methods, Results and Figures (S1-S4);
- an Excel file with supplementary Tables (S1-S13).

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Table 1. Results (p values) of adonis2 PERMANOVA analyses of partial correlation between physiological performance traits and multivariate unweighted (community membership) and weighted (community structure) UniFrac beta-diversity metrics.

	Community membership				Community structure			
	both diet types		SD	LQD	both diet types		SD	LQD
	trait	trait × diet	trait	trait	trait	trait × diet	trait	trait
In both cage types								
MD_{FT}	0.044	0.047	0.167	0.156	0.011	0.037	0.079	0.063
In standard cages (SC)								
MD_{FT}	0.299	0.818	0.972	0.159	0.023	0.154	0.182	0.011
In individually-ventilated cages (IVC)								
MD_{FT}	0.241	0.083	0.065	0.378	0.286	0.170	0.274	0.460
FC	0.004	0.078	0.000	0.143	0.188	0.023	0.049	0.185
ADE	0.000	0.096	0.000	0.001	0.004	0.206	0.003	0.170
FD	0.077	0.125	0.042	0.329	0.253	0.038	0.157	0.187

NOTE The analyses were performed with the same models as these used for testing the effects of selection and experimental factors on the beta-diversity metric, with three additional factors: a covariate representing a performance trait, its interaction with diet, and a covariate representing time of day at the start of the performance trait measurement. MD_{FT} – body mass change in the feeding trial (g/5 days), FC – food consumption rate(g/d); ADE – apparent digestive efficiency (digestibility, %); FD – effective food digestion rate (g/d; a proxy for metabolizable energy intake).

Figure legends

Figure 1 The scheme of the *nature-nurture*, cross-fostering experiment on bank voles from the selection experiment. Voles from four replicate “Control” (C) and four replicate “Herbivorous” (H) lines were cross-fostered after birth. Acronyms CC, CH, HC and HH indicate the combinations of the biological (first letter) and foster mother (second letter) linetypes. Body mass changes and food consumption were measured in two types of cages (SC – standard cages, IVC – individually ventilated metabolic cages), with two types of diet (SD – standard diet, LQD – low-quality diet). Finally, caecal samples were collected for microbiome analyses.

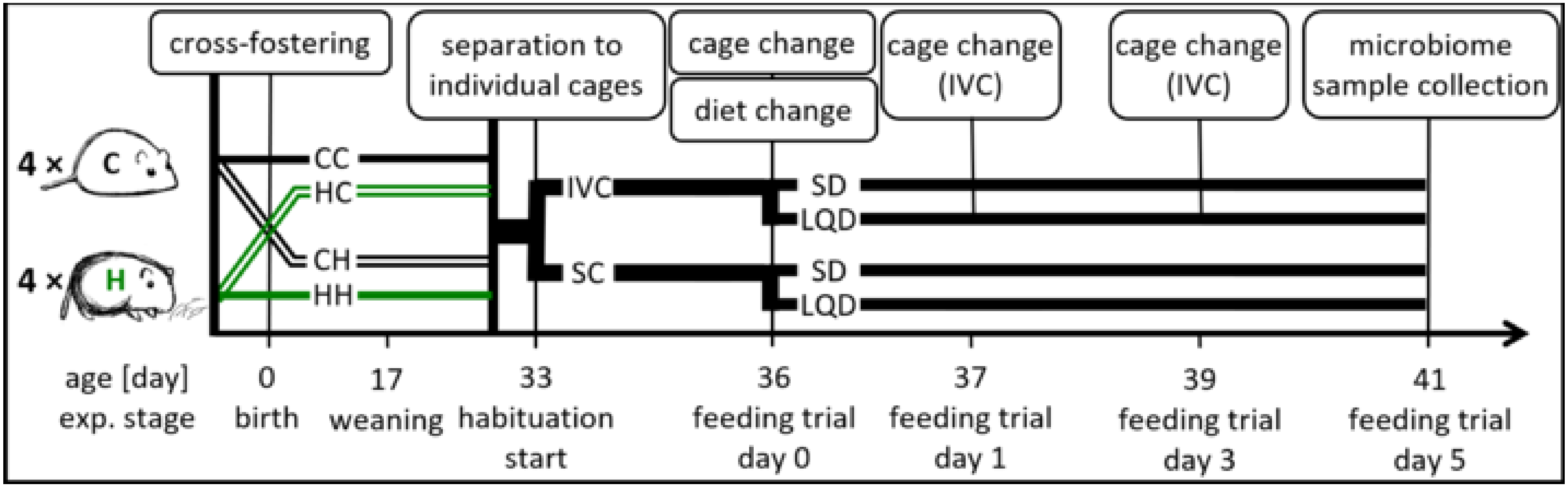
Figure 2 Alpha-diversity metrics of caecal bacterial microbiome in bank voles (least squares means ± 95% CI). The three alpha diversity metrics – the number of amplicon sequence variants (ASV), Shannon index and Pielou index – were computed for subgroups defined by combination of the biological and foster mother linetypes (C – Control, H – Herbivorous) and diet type (SD – standard diet, LQD – low-quality diet), based on rarefied data, and only for *Ci*-free animals, i.e., not hosting bacteria from *Clostridium innocuum* group (results concerning *Ci*-present animals are presented in Figure S2, available online).

Figure 3 Caecum bacterial community characteristics described with a Principal Coordinate Analysis (PCoA), based on UniFrac distances: (**A, B)** unweighted (community membership), and **C, D)** weighted (community structure). The data points are centroids of groups of voles representing four combinations of biological and foster mother line type (see legend), each represented by groups fed the standard (SD) or low-quality diet (LQD), and kept in either standard cages or individually ventilated metabolic cages (cage type is not distinguished on this graph). The first PCoA axis, and in the case of unweighted UniFrac also the second axis **(A, C)**, differentiate primarily between voles fed the SD or LQD diet. Further axes **(B, D)** differentiate also between the combinations of biological and foster mother types. Ovals are added for clarity of the information (they do not show a statistical property). The analyses were performed only for *Ci*-free animals (results concerning *Ci*-present animals are presented in Figure S2, available online).

Figure 4 Relative abundance of A) main bacterial phyla, and B) the most abundant and universal genera (abundance >1% of total microbiome and present in >10% individuals). The values were computed for subgroups defined by combination of the biological and foster mother linetypes (C – Control, H – Herbivorous) and diet type (SD – standard diet, LQD – low-quality diet), based on for rarefied data, and only for *Ci*-free animals (results concerning *Ci*-present animals are presented in Figure S2, available online).

Figure 5 Body mass and performance in the feeding trial (least squares means ± 95% CI). Top row: initial body mass and its change throughout the trial (MD_{FT}); bottom row: the rate of food consumption (FC), apparent digestive efficiency (ADE), and rate of efficient food digestion (FD). The values were computed for subgroups defined by combination of the biological and foster mother linetypes (C – Control, H – Herbivorous), diet type (SD – standard diet, LQD – low-quality diet), and cage type (SC – standard cages, IVC – individually-ventilated metabolic cages).

Figure 6 Correlation between residual values the performance and microbiome traits: (**A-D**) correlation between the rate of food consumption (FC) or apparent digestive efficiency (ADE), and the number of amplicon sequence variants (ASVs) or relative abundance of *Ruminococcus;* (**E, F**) correlation between body mass changes during the feeding trial (MD_{TF}) and relative abundance of Actinobacteriota or *Lactobacillus*. The residuals and partial regression slopes were derived from the ANCOVA models where performance traits were dependent variables, and microbiome variables were included as covariates. All models were corrected for the same set of factors. The analyses were performed for data split by cage type, and excluding outliers specific for the variable in question.


cross-fostering
separation to individual cages
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4 × C
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CC
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SC
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SD
LQD
age [day]
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33
habituation start
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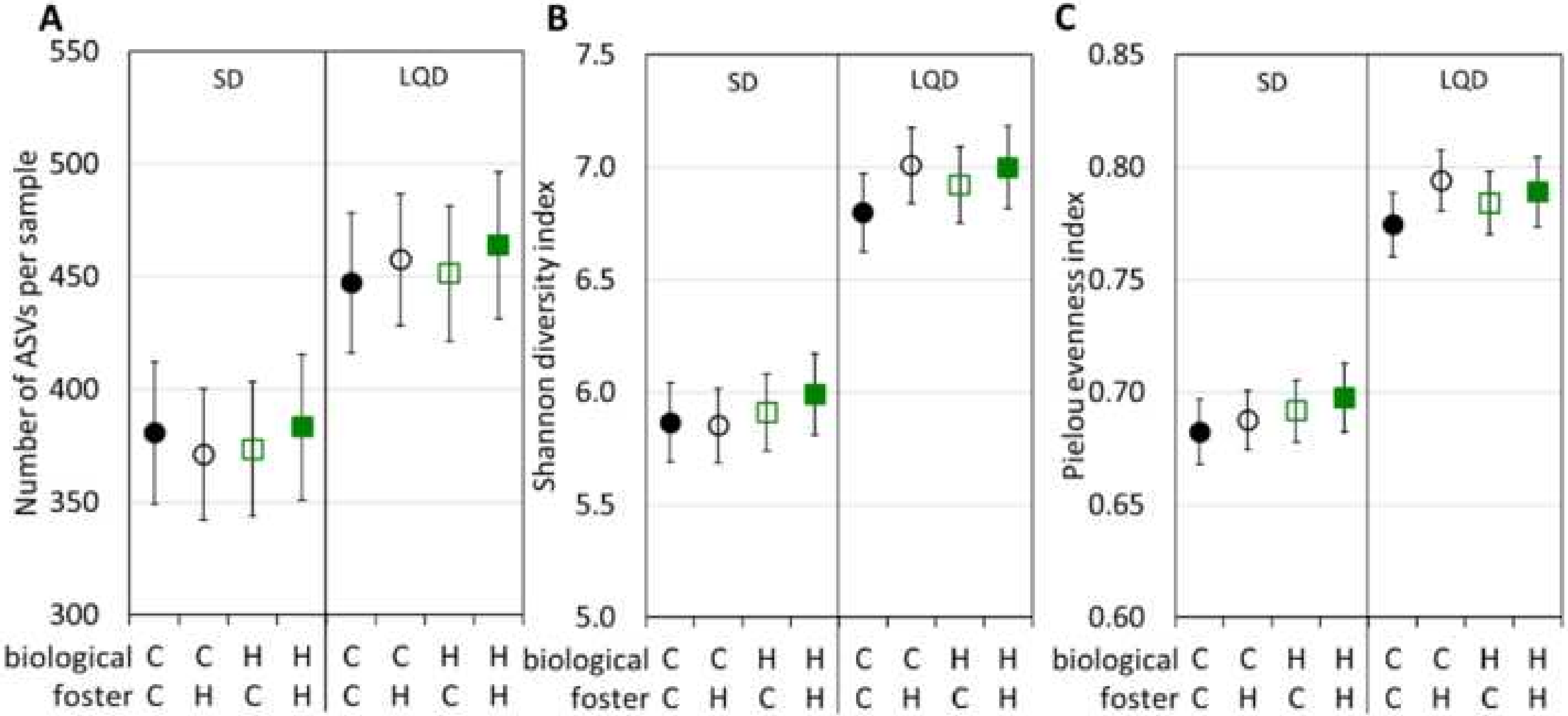
feeding trial day 3
41
feeding trial day 5


A
B
C
Number of ASVs per sample
550
500
450
400
350
300
Shannon diversity index
7.5
7.0
6.5
6.0
5.5
5.0
Pielou evenness index
0.85
0.80
0.75
0.70
0.65
0.60
SD
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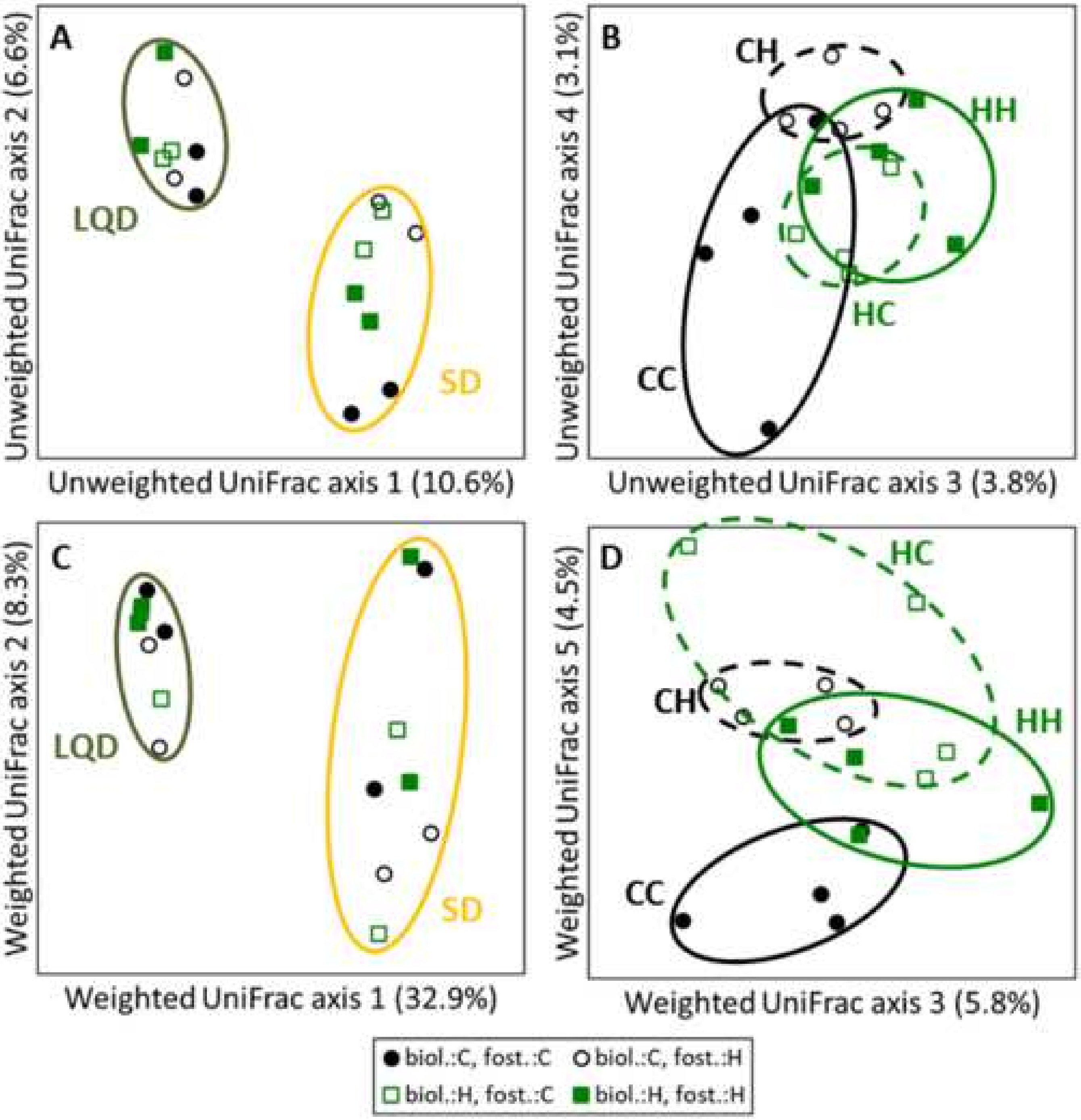
foster C H C H C H C H


A
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Unweighted UniFrac axis 1 (10.6%)
Unweighted UniFrac axis 2 (6.6%)
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CC
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Unweighted UniFrac axis 4 (3.1%)
C
LQD
SD
Weighted UniFrac axis 1 (32.9%)
Weighted UniFrac axis 2 (8.3%)
D
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CH
HH
CC
Weighted UniFrac axis 3 (5.8%)
Weighted UniFrac axis 5 (4.5%)
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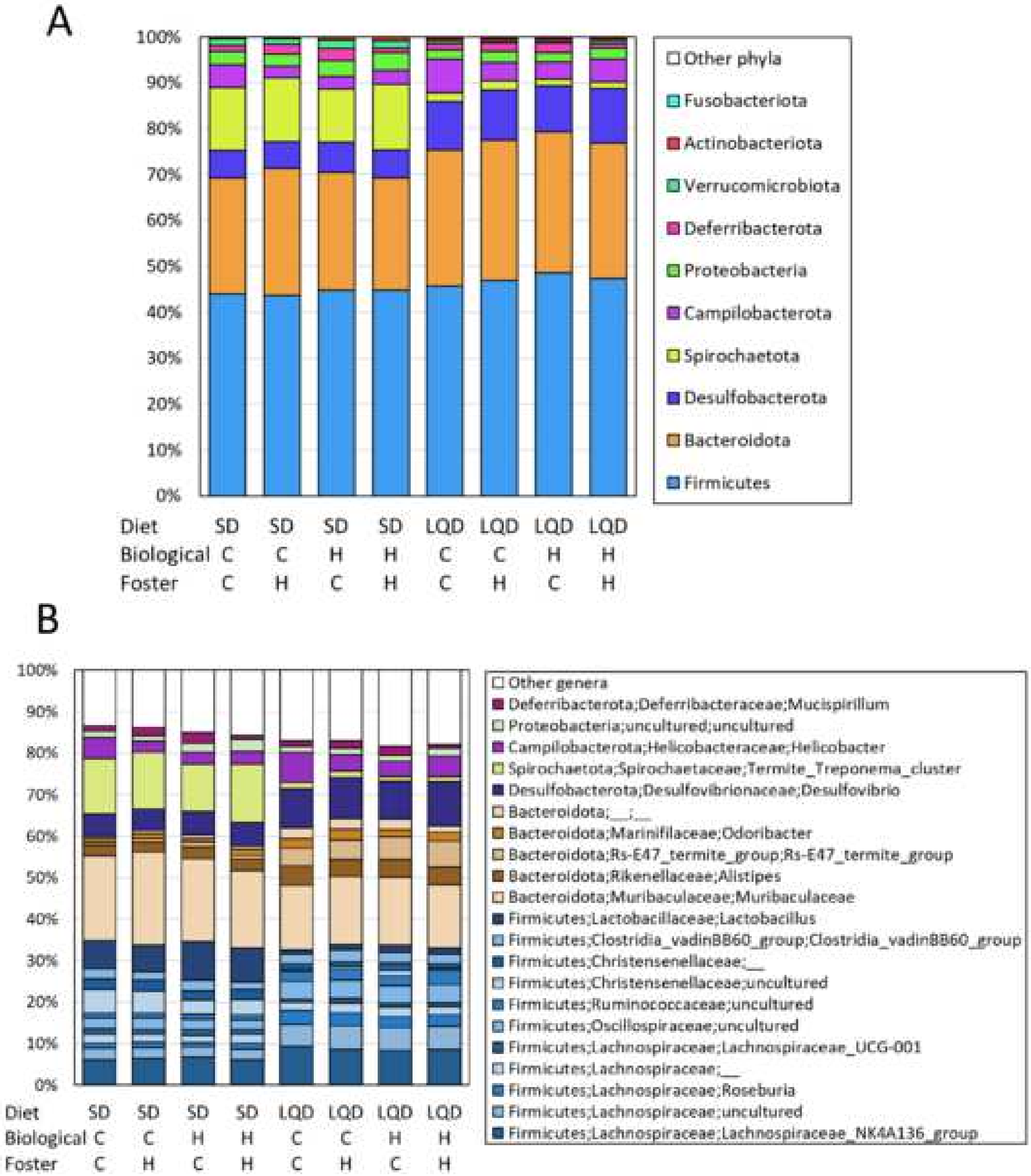
☐ biol.:H, fost.:C
■ biol.:H, fost.:H

Figure 4


A
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Other phyla
Fusobacteriota
Actinobacteriota
Verrucomicrobiota
Deferribacterota
Proteobacteria
Campilobacterota
Spirochaetota
Desulfobacterota
Bacteroidota
Firmicutes
Diet SD SD SD SD LQD LQD LQD LQD
Biological C C H H C C H H
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100%
90%
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Other genera
Deferribacterota;Deferribacteraceae;Mucispirillum
Proteobacteria;uncultured;uncultured
Campilobacterota;Helicobacteraceae;Helicobacter
Spirochaetota;Spirochaetaceae;Termite_Treponema_cluster
Desulfobacterota;Desulfovibrionaceae;Desulfovibrio
Bacteroidota;__;__
Bacteroidota;Marinifilaceae;Odoribacter
Bacteroidota;Rs-E47_termite_group;Rs-E47_termite_group
Bacteroidota;Rikenellaceae;Alistipes
Bacteroidota;Muribaculaceae;Muribaculaceae
Firmicutes;Lactobacillaceae;Lactobacillus
Firmicutes;Clostridia_vadinBB60_group;Clostridia_vadinBB60_group
Firmicutes;Christensenellaceae;__
Firmicutes;Christensenellaceae;uncultured
Firmicutes;Ruminococcaceae;uncultured
Firmicutes;Oscillospiraceae;uncultured
Firmicutes;Lachnospiraceae;Lachnospiraceae_UCG-001
Firmicutes;Lachnospiraceae;__
Firmicutes;Lachnospiraceae;Roseburia
Firmicutes;Lachnospiraceae;uncultured
Firmicutes;Lachnospiraceae;Lachnospiraceae_NK4A136_group
Diet SD SD SD SD LQD LQD LQD LQD
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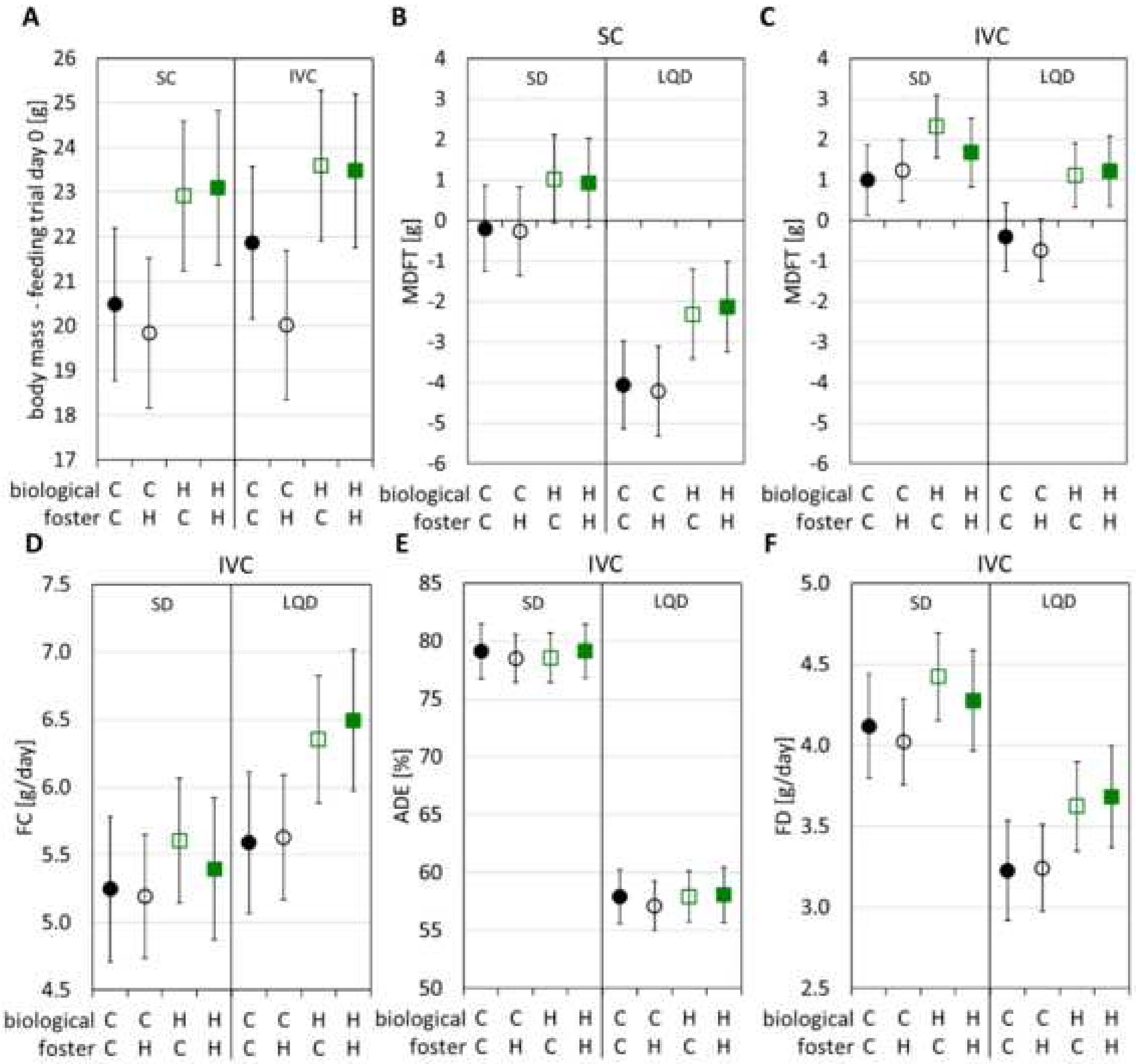
A
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body mass - feeding trial day 0 [g]
26
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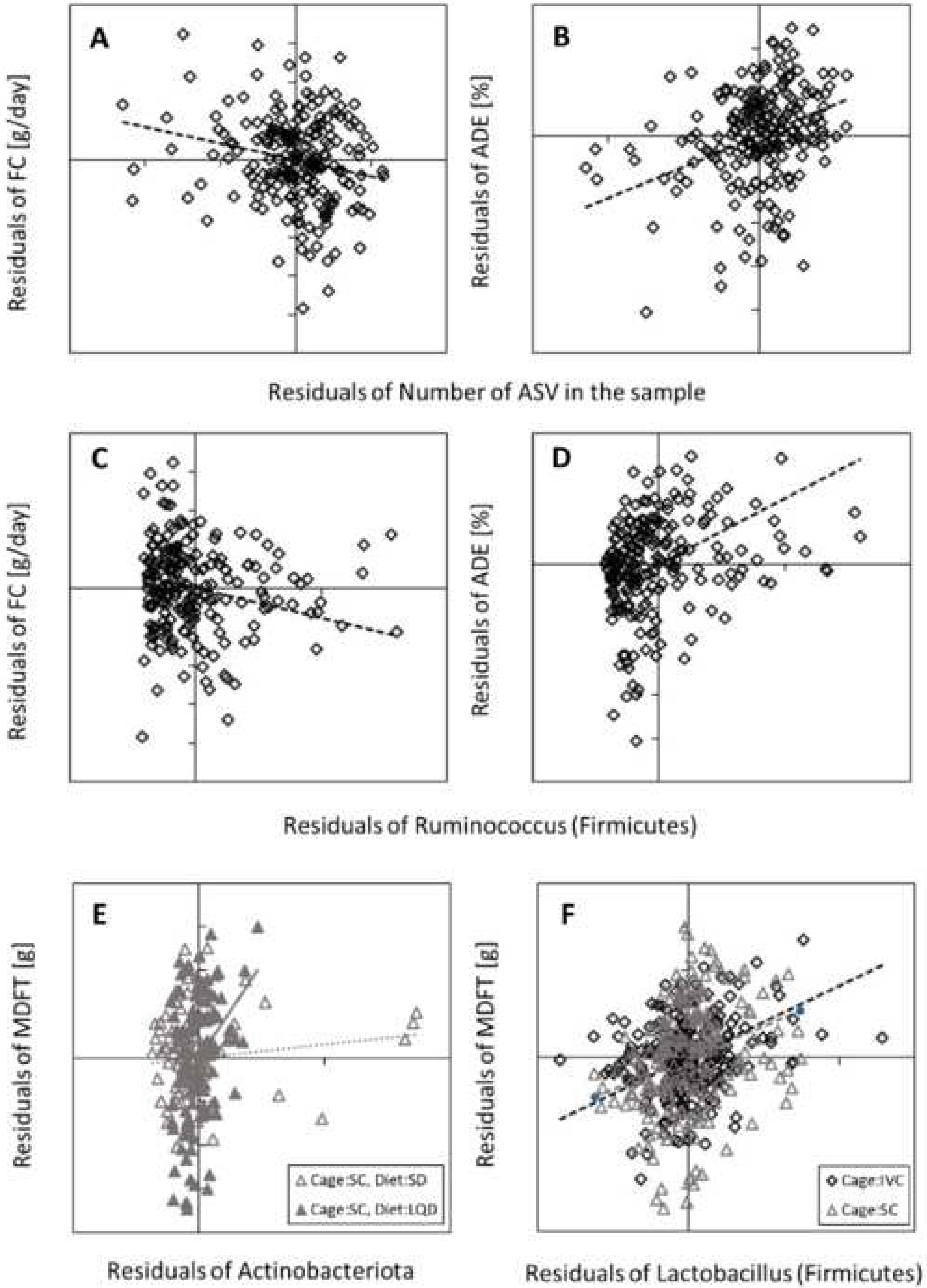
4.5
ADE [%]
85
80
75
70
65
60
55
50
FD [g/day]
5.0
4.5
4.0
3.5
3.0
2.5

FIgure 6


A
Residuals of FC [g/day]
B
Residuals of ADE [%]
Residuals of Number of ASV in the sample
C
Residuals of FC [g/day]
D
Residuals of ADE [%]
Residuals of Ruminococcus (Firmicutes)
E
Residuals of MDFT [g]
△ Cage:SC, Diet:SD
▲ Cage:SC, Diet:LQD
Residuals of Actinobacteriota
F
Residuals of MDFT [g]
◇ Cage:IVC
△ Cage:SC
Residuals of Lactobacillus (Firmicutes)