Limited changes in the fecal microbiome composition of laying hens after oral inoculation with wild duck feces

Janneke Schreuder [*,1] Francisca C. Velkers [*] Ruth J. Bouwstra,[†] Nancy Beerens [‡] J. Arjan Stegeman,[*] Willem F. de Boer,[§] Armin R. W. Elbers [‡] Pim van Hooft,[§] Anneke Feberwee,[†] Alex Bossers [‡] and Stephanie D. Jurburg [‡,#]

[*]*Department of Farm Animal Health, Utrecht University, 3584 CL, the Netherlands;* [†]*GD Animal Health, 7418EZ Deventer, the Netherlands;* [‡]*Wageningen Bioveterinary Research, 8221RA Lelystad, the Netherlands;* [§]*Resource Ecology Group, Wageningen University & Research, 6708PB Wageningen, the Netherlands; and* [#]*German Centre for Integrative Biodiversity Research (iDiv), Halle-Jena-Leipzig 04103, Germany*

ABSTRACT Interspecies transmission of fecal microbiota can serve as an indicator for (indirect) contact between domestic and wild animals to assess risks of pathogen transmission, e.g., avian influenza. Here, we investigated whether oral inoculation of laying hens with feces of wild ducks (mallards, *Anas platyrhynchos*) resulted in a hen fecal microbiome that was detectably altered on community parameters or relative abundances of individual genera. To distinguish between effects of the duck inoculum and effects of the inoculation procedure, we compared the fecal microbiomes of adult laying hens resulting from 3 treatments: inoculation with wild duck feces (duck), inoculation with chicken feces (auto), and a negative control group with no treatment. We collected cloacal swabs from 7 hens per treatment before (day 0), and 2 and 7 D after inoculation, and performed 16S rRNA amplicon sequencing. No distinguishable effect of inoculation with duck feces on microbiome community (alpha and beta diversity) was found compared to auto or control treatments. At the individual taxonomic level, the relative abundance of the genus *Alistipes* (phylum *Bacteroidetes*) was significantly higher in the inoculated treatments (auto and duck) compared to the control 2 D after inoculation. Seven days after inoculation, the relative abundance of *Alistipes* had increased in the control and no effect was found anymore across treatments. These effects might be explained by the perturbation of the hen's microbiome caused by the inoculation procedure itself, or by intrinsic temporal variation in the hen's microbiome. This experiment shows that a single inoculation of fecal microbiota from duck feces to laying hens did not cause a measurable alteration of the gut microbiome community. Furthermore, the temporary change in relative abundance for *Alistipes* could not be attributed to the duck feces inoculation. These outcomes suggest that the fecal microbiome of adult laying hens may not be a useful indicator for detection of single oral exposure to wild duck feces.

Key words: laying hen, fecal microbiota, wild duck, 16S rRNA gene sequencing, inoculation

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INTRODUCTION

Contact between wildlife and domestic animals can lead to transmission of pathogens, as wildlife can serve as a reservoir host (Gortázar et al., 2007). The interaction between wild birds and poultry has become more important in recent years, because of the increased demand for free-range poultry products whereby outdoor access for poultry increases the risk to pathogen exposure originating from wild birds (Koch and Elbers, 2006). It is therefore important to have alternative methods available to study transmission of infectious agents between wild birds and poultry to facilitate risk assessment and develop preventive measures to reduce potential risks for transmission of infectious diseases.

The most striking example of potential risks associated with the wild bird-poultry interface is avian influenza virus (**AIV**) outbreaks in poultry farms. Wild migratory birds play an important role in the spread of both low pathogenic AIV and highly pathogenic AIV across continents (Lycett et al., 2016). The close genetic relationship between AIV in waterfowl and domestic poultry in several outbreaks supports the role of wild waterfowl in outbreaks (Munster et al., 2005; Berhane et al., 2009; Lebarbenchon and Stallknecht, 2011; Beerens et al., 2018).

In waterfowl, low pathogenic AIV is most often detected in mallards (*Anas platyrhynchos*) (Verhagen et al., 2017). Moreover, video-camera monitoring at

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[1]Corresponding author: j.schreuder@uu.nl

a Dutch poultry farm showed that mallards were frequent occupants of the outdoor range at night between November and March (Elbers, 2017). As AIV is shed in high concentrations in feces of infected birds (França et al., 2012), infected waterfowl in the vicinity of outdoor ranges can contaminate the farm environment. Depending on environmental conditions, the virus may persist in the environment for many months (Brown et al., 2007; Stallknecht and Brown, 2017). Chickens can become infected directly via coprophagic behavior (Hyun and Sakaguchi, 1989; von Waldburg-Zeil et al., 2019), or indirectly via contact with an environmental virus reservoir (Brown et al., 2007; Rohani et al., 2009).

As the role of waterfowl in the transmission of infectious agents to poultry is mainly linked to fecal contamination (Swayne and Pantin-Jackwood, 2006), studying transmission of fecal microbiota between waterfowl and chickens may reveal proxies for contact between them. Previously, the genetic subtypes of gut-residing *Escherichia coli* served as a proxy for contact between giraffes and wild primates (VanderWaal et al., 2014; Springer et al., 2016) or for pathogen transmission between individuals (Blyton et al., 2014). Song et al. (2013) showed that humans in the same household shared fecal microbiota. If dogs were present in the household, humans also shared certain skin microbiota with the dogs. In wild baboons, social group membership and social network relationships predicted the taxonomic structure of the gut microbiome, and rates of social interaction directly explained variation in the gut microbiome (Tung et al., 2015). Similarly, the fecal microbiota of chickens may be affected by the presence of waterfowl. Thus, the chicken's fecal microbiome may be used to assess whether contact with waterfowl feces has occurred. If changes in the fecal microbiome can be determined in chickens, then this may serve as a proxy for the risk of pathogen transmission, e.g., AIV, prior to actual outbreaks, and can be used for risk assessment purposes.

In this study, we investigated the transmissibility of fecal microbiota from wild mallard feces (further referred to as duck) to antibiotic-free recipient laying hens in the week following an oral inoculation with these duck feces. In medicine, fecal microbiota transplants (**FMT**) are applied to human subjects as a treatment for gut dysbiosis, for instance in patients with *Clostridium difficile* infection (Hamilton et al., 2012; Cammarota et al., 2017). Although a treatment with antibiotics is often applied before FMT, Li et al. (2016) showed that FMT could also be successful in antibiotic-free patients with metabolic syndrome. We hypothesized that duck fecal microbiota can be transmitted to laying hens via oral inoculation with duck feces, causing detectable shifts in the fecal microbiome composition of laying hens by altering the whole microbial community or the relative abundance of specific bacterial taxa. To distinguish between the effects of the feces inoculation process and the specific effects of the duck feces inoculation, we compared the fecal microbiomes resulting from duck feces inoculation to those resulting from an inoculation with chicken feces (auto inoculation) and a negative control group. In particular, we expected to detect novel taxa in the hen feces after inoculation which were present in the duck inoculum, or altered dominance patterns resulting in an altered community in the hen microbiome after inoculation.

MATERIALS AND METHODS

Ethics

The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Wageningen University and Research, the Netherlands. The animal experiments were executed at the Dutch Animal Health Service (GD Deventer, the Netherlands) and were done in full compliance with all relevant legislation. The capture of free-living birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2014/054).

Hens, Management, and Experimental Design

A total of 54 Bovans Brown laying hens of 19 wk of age were obtained from a commercial pullet-rearing farm and transported to the experimental facility. Upon arrival, the hens were placed in a 3-tiered aviary system. The tiers were divided by plastic partitions, and cages on the same tier were separated by wire fences, with wood shavings covering the ground. The hens had a habituation period of 12 wk prior to the start of the experiment and were subjected to a standard light regime for laying hens. A commercial layer feed (ABZ Diervoeding, Nijkerk) without antibiotics and water was supplied ad libitum. The animals were observed daily and the presence of clinical signs or abnormal behavior, and mortality was recorded.

To study the transmission of fecal microbiota from wild ducks to laying hens, we subjected the hens to one of 3 treatments: a single oral inoculation with an inoculum made from wild duck feces (**duck** treatment); a single oral inoculation with an inoculum made from the feces of the recipient laying hens (**auto** treatment); and a negative control without any treatment (**control** treatment). Each treatment group consisted of 18 laying hens.

Inoculum Preparation, Inoculation, and Sample Collection

Fresh fecal droppings of mallards were collected opportunistically during avian influenza surveillance activities in wild birds in the Netherlands, as routinely performed by Erasmus Medical Center (Rotterdam, the Netherlands). Fecal droppings of a maximum of 3 wild

ducks were pooled (a batch). In total, 39 batches with fecal droppings from 104 wild ducks were collected over 2 sampling days. The fecal batches were immediately stored on ice, and processed on the day of collection. The batches were processed separately to prevent cross contamination between batches with AIV. Prior to further processing, all batches were tested by PCR on AIV (Bouwstra et al., 2015) and *Salmonella* (Halatsi et al., 2006) at GD Deventer (the Netherlands). Batches which tested positive for AIV or *Salmonella* (18 batches in total) were excluded from further processing to prevent introduction of these pathogens into the experimental facilities. Fresh fecal droppings of all chickens in the research facilities were collected and processed as a single pool.

The pool of chicken feces and the duck fecal batches were prepared according to the protocol described by Youngster et al. (2014) with slight modifications: batches with duck fecal droppings were diluted 1:1 and pooled chicken fecal droppings were diluted 1:2 with sterile PBS (DPBS, Gibco, ThermoFisher Scientific, the Netherlands). The mixtures were thoroughly homogenized, and large particles were removed by passing through a sterile 0.7 mm sieve. Of these fecal suspensions, 5 samples of the chicken inoculum and 5 samples of each duck batch were stored in −80°C for DNA extraction and 16S rRNA gene sequencing at a later stage. The suspension was centrifuged at 3,000 rpm for 30 min, and the obtained pellet was suspended in sterile PBS with 20% glycerol (BioXtra >99% GC, Sigma Aldrich, the Netherlands). The final fecal concentration in all inocula was approximately 1 g of pooled feces in 1 mL of PBS + 20% glycerol. Bacterial viability was checked for all inocula by quantifying colony-forming units using blood agar plates (Supplemental Table S1). All inocula were stored at −80°C and were thawed at 4°C for 12 h prior to further processing at day of inoculation.

At the day of inoculation (day 0), 10 batches with fecal duck inocula were combined and homogenized to form 1 duck inoculum. Subsequently, the chickens (31 wk of age) were inoculated with 6 mL of either the duck or auto inoculum via oral gavage. The negative control group remained untreated. Two cloacal swabs per hen were collected daily for all chickens from day 0 (prior to inoculation) until the end of the experiment at day 13. Cloacal swabs were stored on ice upon collection and stored at −80°C within 2 h. On day 13, all chickens were euthanized by intravenous injection with a 20% pentobarbital-sodium solution.

DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Per treatment, 7 chickens, from a total of 18 chickens, were selected for further analysis of samples taken on a subset of timepoints, i.e., 0, 2, and 7 D after inoculation. Cloacal swabs of 7 chickens were selected and visually assessed to ensure that sufficient fecal material for DNA extraction was available on the swabs on all 3 selected timepoints. Based on microbiota studies in laying hens and broilers, a sample size of 7 cloacal swabs per treatment group was expected to be large enough to detect differences in microbiota composition with sufficient statistical power (Videnska et al., 2014b; Jurburg et al., 2019). Day 0 was chosen as a reference baseline, and we expected to measure the first shift in the fecal microbiome 2 D after inoculation. The last timepoint chosen for analyses was day 7 after inoculation was included to determine if shifts in the fecal microbiota composition found on day 2 were still detectable. For each time × chicken combination, the duplicate swab samples were used for DNA extraction to ensure sufficient DNA was obtained for sequencing. Five of the duck and chicken fecal suspensions (**Inoduck** and **Inochicken** respectively), which were stored at −80°C during inoculum preparation, were used for DNA isolation. Swabs were thawed at room temperature, diluted in 1 mL of sterile PBS, and vortexed for 15 s. DNA was extracted from 200 μL of these diluted fecal suspension or cloacal swab samples using the Qiagen QIAamp Fast DNA stool mini kit (Qiagen, Hilden, Germany) and processed according to the manufacturer's instructions, with an additional bead-beating step. DNA extracts were quantified with Invitrogen Qubit 3.0 Fluorometer and stored at −20°C for further processing. DNA from duplicate swab samples was pooled after extraction.

The V3–4 region of the 16S rRNA gene was amplified in a PCR with the primers CVI_V3-forw CCTACGGGAGGCAGCAG and CVI_V4-rev GGACTACHVGGGTWTCT. The following amplification conditions were used: step 1: 98°C for 2 min, step 2: 98°C for 10 s, step 3: 55°C for 30 s, and step 4: 72°C for 10 s, step 5: 72°C for 7 min. Steps 2 to 4 were repeated 25 times. PCR products were checked with gel electrophoresis, and PE300 sequencing was performed using a MiSeq sequencer (Illumina Inc., San Diego, CA).

Processing of Sequencing data

All sequence processing and statistical analyses were performed in R 3.5.1 (R Core Team 2018). The sequenced reads were filtered, trimmed, dereplicated, chimera-checked, and merged using the dada2 package (Callahan et al., 2016) using standard parameters (TruncLength = 240, 210), and reads were assigned with the SILVA v.132 classifier (Quast et al., 2012). Downstream analyses were performed with the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2007) R packages. Good's coverage was >0.999. Prior to all analyses, the data were rarefied to 2,658 reads per sample (rarefy_even_depth, seed = 1), to standardize the number of reads while preserving all samples. The final dataset contained 1,193 amplicon sequence variants.

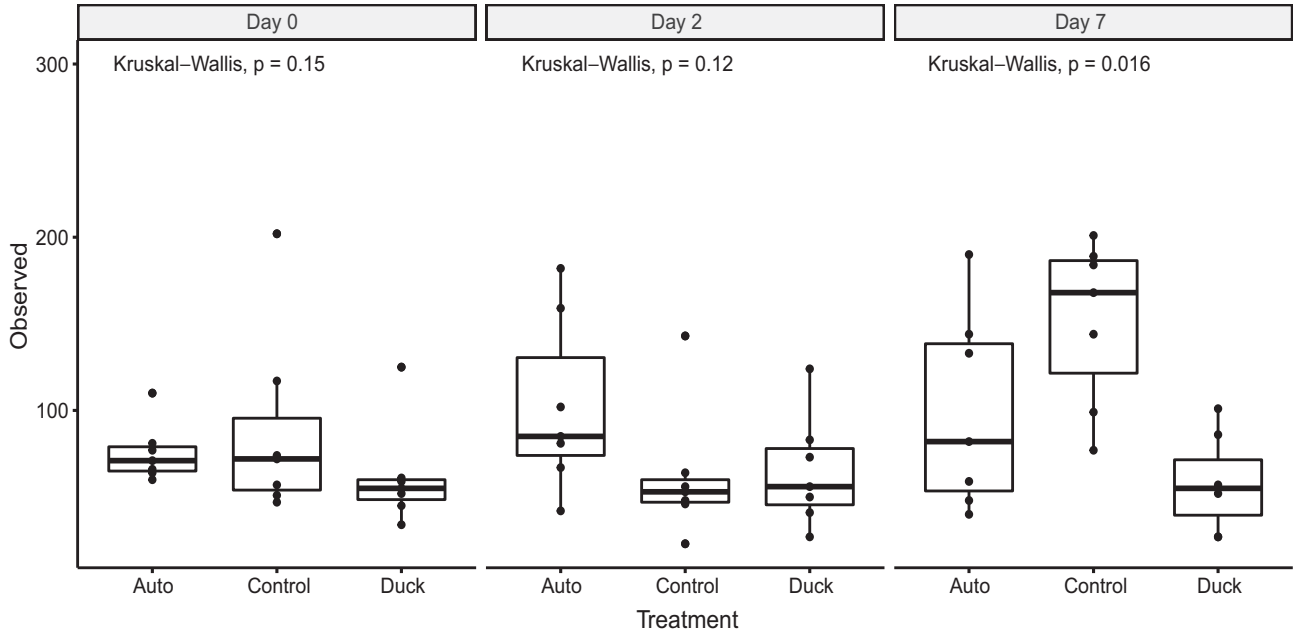

Figure 1. Observed species richness in all 3 treatments and grouped per day. Each dot represents an individual laying hen. Kruskal–Wallis test was used to detect significant differences in alpha diversity across treatment groups per day. The chicken inoculum (InoChicken; n = 5) exhibited mean observed species richness of 166 ± 6.2, and the duck inoculum (InoDuck; n = 5) exhibited mean observed species richness of 112.2 ± 36.44 (results are not shown). Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

Statistical Analysis

The number of amplicon sequence variants per sample was used as a measure of observed richness (alpha diversity). To test for effects of inoculation on richness, Kruskal–Wallis tests were performed per time x treatment. To evaluate whether the duck inoculation had an effect on the bacterial community composition, principal coordinate analysis of Bray–Curtis distances was used to visualize differences in microbiome community structure across treatments and over time. Clustering patterns of samples were assessed visually, and the statistical significance was confirmed with a PERMANOVA-like adonis on Bray–Curtis distances from the vegan package. Homogeneity of variances in microbial communities between samples from the same time x treatment combination was measured with betadisper from the vegan package. To examine if inoculation with duck feces had an effect on the relative abundance of specific genera compared to the auto inoculation and control on the samples taken 2 and 7 D after inoculation, we performed Kruskal–Wallis tests on genera with an average relative abundance of at least 0.5%. Genera for which $P < 0.05$ were selected for further analysis. To further disentangle effects of the inoculation procedure itself vs. actual inoculation with duck feces, we checked for significant differences in selected genera between inoculated (duck + auto) vs. control and between duck and auto treatments with Wilcoxon rank-sum tests. These genera were plotted in ternary plots per timepoint using R ggtern package (Hamilton and Ferry, 2018). Relative abundances in taxa over time are reported throughout the manuscript as mean ± SD.

RESULTS

Community Level Changes

To characterize the microbial community of the laying hen's fecal microbiome, we first explored community diversity. Observed species richness (alpha diversity) exhibited no significant differences across treatments on days 0 and 2 (Figure 1). On day 7 after inoculation, there was a lower diversity in the inoculated hens (auto and duck) compared to the control hens (Wilcoxon rank-sum, $P = 0.011$). No significant difference was found between the auto and duck treatments on day 7 (Wilcoxon rank-sum test, $P = 0.20$). There were also no significant differences within treatments over time (Kruskal–Wallis test, $P > 0.05$). A principal coordinate analysis of the Bray–Curtis distances (beta diversity) was used to evaluate the changes in community structure across treatments and over time (Figure 2). Samples did not show any significant clustering ($P > 0.05$) of hen samples according to their treatment on day 2 or 7 after inoculation (Supplemental Table S2). Prior to inoculation on day 0, a significant difference in community structure was detected between the control and duck treatments (PERMANOVA-like Adonis, $P = 0.048$). No significant clusters were observed within treatments over time ($P > 0.05$).

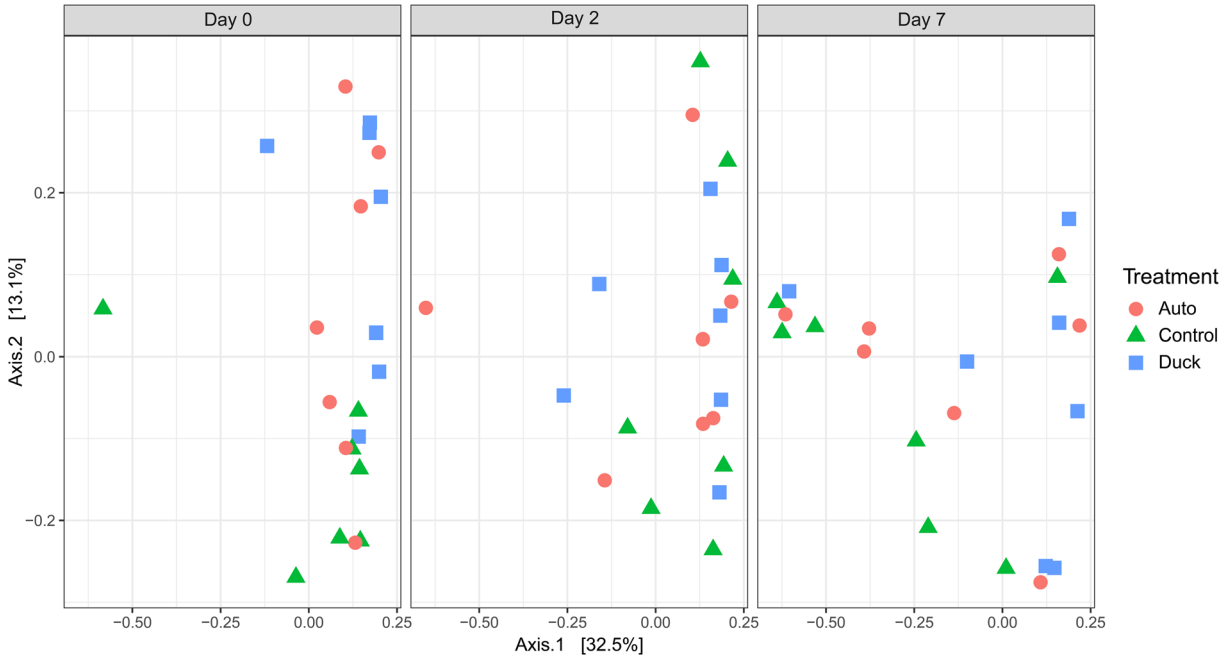

Figure 2. Change in community composition of the fecal microbiomes in hens visualized in a single PCoA plot of Bray–Curtis distances per treatment group and faceted per day. Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

Bacterial Composition and Temporal Dynamics

In order to examine the dynamics in specific phyla and genera between treatment groups and over time, we selected the 10 most abundant phyla and 15 most abundant genera among all samples collected in the study on average. Average relative abundances are ± standard deviation. At the phylum level, the relative abundance of inocula consisted mainly of the phyla *Firmicutes* (Inoduck 49.2 ± 5.3%; Inochicken 40.0 ± 0.9%) and *Bacteroidetes* (Inoduck 27.6 ± 3.8%; Inochicken 45.5 ± 0.9%). At the genus level, the relative abundance of Inoduck was dominated by *Megamonas* (15.7 ± 2.4%, phylum *Firmicutes*) and *Bacteroides* (14.2 ± 4.1%, phylum *Bacteroidetes*) (Supplemental Figure S1). Inochicken was dominated by *Rikenellaceae_RC9_gut_group* (13.0 ± 1.1%) followed by *Bacteroidales* (7.2 ± 0.6%), *Alistipes* (7.0 ± 0.8%), and *Bacteroides* (6.7 ± 0.6%), all belonging to the phylum of *Bacteroidetes* (Supplemental Figure S1). All other genera showed relative abundances below 6% in the inocula.

In the fecal samples of the hens, *Firmicutes* was dominant across all treatment groups and timepoints (66.4 ± 12.8%). *Fusobacteria* had a much lower abundance (10.5 ± 12.7%). All other phyla exhibited relative abundances <10% (Figure 3). At the genus level (Figure 4), *Romboutsia* (19.8 ± 12.3%, phylum *Firmicutes*) and *Fusobacterium* (10.5 ± 12.6%, phylum *Fusobacteria*) were most abundant across all treatments and timepoints (Figure 4). Although highly present in the duck inoculum (15.7 ± 2.4%), *Megamonas* was not observed in the fecal samples of the hens.

To further explore phyla and genera which showed consistent differences across treatment groups (Kruskal–Wallis test, $P < 0.05$), we selected the 10 most abundant phyla, and genera with an average relative abundance of > 0.5% at 2 and 7 D after inoculation, resulting in 1 phylum and 7 genera for further analyses. The phylum *Bacteroidetes* had a higher relative abundance in the inoculated treatments (duck and auto) compared to the control (Wilcoxon rank-sum test, $P = 0.028$ and $P = 0.014$ respectively) 2 D after inoculation. Of the 7 genera, 5 were present in the duck inoculum, and 4 of these (*Alistipes, Bacteroides, Faecalibacterium, and Ruminiclostridium 9*) had a lower relative abundance ($P < 0.05$) prior to inoculation (Supplemental Table S3). *Alistipes* exhibited a higher (Wilcoxon rank-sum test, $P = 0.009$) relative abundance in samples from the inoculated treatments (auto and duck) compared to the control 2 D after inoculation, with no difference between the duck and auto treatments. The relative abundance of *Alistipes* was higher (Wilcoxon rank-sum test, $P = 0.035$) 2 D after inoculation than before inoculation in both the auto and duck treatments. However, 7 D after inoculation, the relative abundance of *Alistipes* in the auto and duck treatments was similar to the control (Wilcoxon rank-sum test, $P = 0.12$). No significant changes in the relative abundances of *Bacteroides, Faecalibacterium,* and *Ruminiclostridium 9* were detected in the inoculated hens (duck and auto) over time, nor were there significant differences in the relative abundances of these

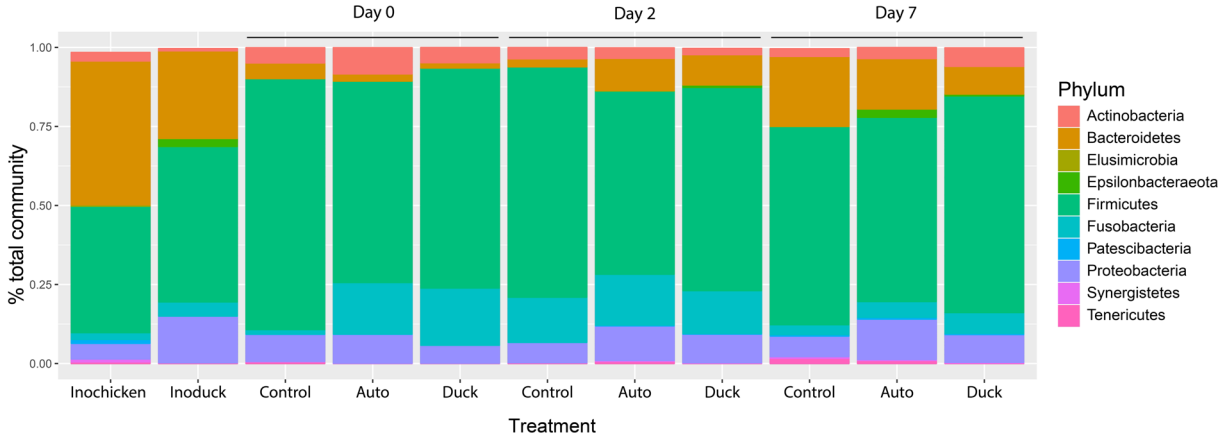

Figure 3. Relative abundance (%) of ten most abundant phyla displayed per treatment group and faceted per timepoint. Average values per inoculum, and treatment and timepoint are displayed. Inochicken: chicken inoculum (n = 5). Inoduck: duck inoculum (n = 5). Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

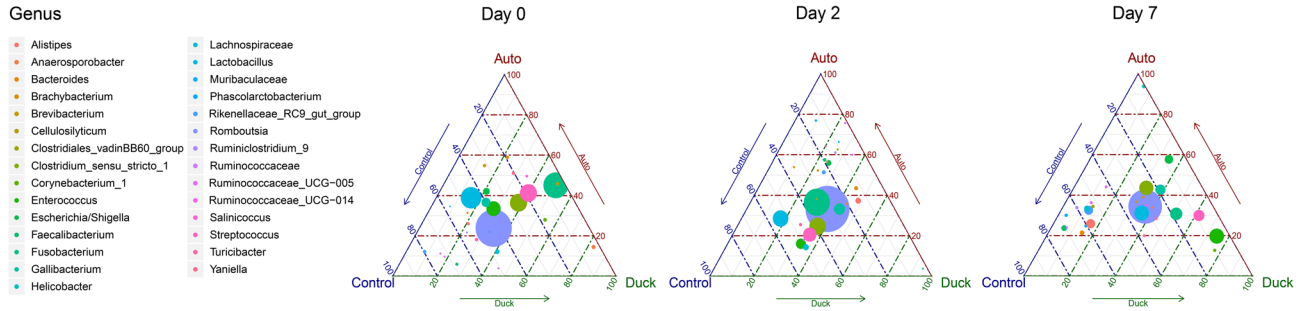

Figure 4. Ternary plot of genera with average relative abundance >0.5% per timepoint. The plot shows the proportion of the abundance of the genera per treatment group as positions in the triangle using barycentric coordinates. The 3 treatment groups are displayed on the 3 axes of the plot: control in blue, auto in red, and duck in green. The size of the circle indicates the relative abundance for that genus. Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

genera between auto and duck treatments either 2 or 7 D after inoculation ($P > 0.05$). Although not present in the duck inoculum, *Enterococcus* (phylum *Firmicutes*) exhibited higher relative abundances in the duck treatment compared to the auto treatment 2 D after inoculation (Wilcoxon rank-sum test, $P = 0.03$), but not compared to the control ($P = 0.44$). Seven days after inoculation, the relative abundance of *Enterococcus* was higher (Wilcoxon rank-sum test, $P = 0.03$) in the duck treatment compared to the control, but there was no significant difference in relative abundance of *Enterococcus* between duck and auto treatments (Wilcoxon rank-sum test, $P = 0.074$). Although not significant, the relative abundance of *Enterococcus* increased over time in the duck treatment (Kruskal–Wallis test, $P = 0.068$), but decreased significantly over time in the control (Kruskal–Wallis test, $P < 0.01$).

DISCUSSION

Identification of a proxy for the direct or indirect contact between domestic and wild animals may provide more insight into potential effects of these interactions and shed light on the mechanisms of pathogen transmission. This proxy could be used for risk assessment and identification of potential preventive measures to help reduce risks for disease outbreaks. In the present study, we investigated whether an oral inoculation of laying hens with duck fecal microbiota resulted in a hen fecal microbiome that was detectably altered. We hypothesized that the inoculation would result in changes in microbial community parameters (community diversity, community structure) as well as changes in the relative abundance of individual genera that might serve as an indicator for contact between ducks and laying hens.

The microbiome composition of the fecal and inoculum samples was markedly different. However, this was to be expected as fecal swabs and inocula are different matrixes, and the collection and processing after collection differed. Therefore, the samples are not directly comparable. Rather the inocula samples were meant to serve as a general reference for the types of shift that we could expect.

We were not able to detect significant differences in community diversity in the fecal microbiomes of hens inoculated with duck feces compared to hens inoculated with auto treatment or controls. However, we

found that the relative abundance of the genus *Alistipes* (phylum *Bacteroidetes*) was significantly higher in the inoculated treatments (auto and duck) compared to the control at 2 D after inoculation. Previous studies also reported an increase in relative abundance of *Alistipes* after FMT in humans (Low et al., 2012; Hamilton et al., 2013; Lee et al., 2017), which was thought to be associated with colonization properties of bacteria from the order *Bacteroidales* (Lee et al., 2017). However, 7 D after inoculation, the relative abundance of *Alistipes* had also increased in the control group, and no significant differences were detected between any of the treatments. Thus, it is also possible that the significant difference 2 D after inoculation was a result of the intrinsic temporal variation of the microbiome (Li et al., 2016; Fu et al., 2019).

Alternatively, the patterns observed for *Alistipes* may have been a result of the inoculation and sampling procedures, which may have been stressful, and thus affected the microbiome composition (De Palma et al., 2014; Li et al., 2017). It has been shown that *Alistipes* was higher in fecal samples of mice that were exposed to daily stress compared to a non-stressed control group (Li et al., 2017). In addition, the increase in the relative abundance of *Alistipes* in the control treatment may have been a result of transmission of *Alistipes* from inoculated hens. Humans and animals that live together are known to exchange microbiota (Song et al., 2013; Schloss et al., 2014). In animal studies, a cage effect is especially likely to occur for animals that are coprophagic such as mice (McCafferty et al., 2013; Laukens et al., 2016) and chickens (Kers et al., 2018; von Waldburg-Zeil et al., 2019). To avoid cage effects in chickens and to prevent the intake of particles and feathers containing potential intestinal microbiota "contaminants" (Meyer et al., 2012), studies have previously used individual housing of chickens (Zhao et al., 2013). For the purpose of this experiment, we decided not to house animals separately because this would be an additional stress factor for the birds, and would not be representative for the field situation. Therefore, all treatment groups were housed and handled in the same research unit. Consequently, transmission of *Alistipes* (and potentially other genera) from inoculated to control hens cannot be ruled out.

Curiously, the change in the relative abundance of *Alistipes* was the only significant alteration. Numerous studies have been published about the successful colonization of donor microbiota in recipients after FMT in humans (Hamilton et al., 2013; Broecker et al., 2016; Li et al., 2016; Lee et al., 2017; Moss et al., 2017) and other animals (Diao et al., 2016; De Palma et al., 2017; Siegerstetter et al., 2018). In humans, FMTs can be administered orally (Youngster et al., 2014), but are often preceded by preparatory antibiotic treatment or bowel cleansing, which means that the gut microbiome at the time of FMT was disturbed (Manichanh et al., 2010; Dethlefsen and Relman, 2011), making it difficult to disentangle effects of FMT vs. preparatory treatments (Schmidt et al., 2018). In animal studies, young (Volf et al., 2016; Hu et al., 2018; Siegerstetter et al., 2018) or germ-free animals (Diao et al., 2016; De Palma et al., 2017) are often used for FMT. Volf et al. (2016) showed that a single inoculation of newly hatched ISA Brown pullets with cecal contents from donor hens of different ages could establish long-lasting measurable shifts in the cecal microbiota composition. However, in all these studies, the animals and chickens did not have fully developed gut microbiota. As we attempted to find a proxy for contact between wild ducks and adult laying hens with outdoor range, we did not want to use younger hens as recipients of the duck inoculum nor did we want to use a preparatory treatment.

In a previous study, Videnska et al. (2014b) found that the cecal microbiome of laying hens underwent several successional changes in the process of aging. The age of the hens used in this study was 31 wk, which is categorized as the fourth stage (28 to 52 wk). At this stage, the gut microbiome has reached an adult microbial equilibrium (Videnska et al., 2014b). A stable microbiome forms a complex ecosystem and is characterized by a capacity for self-regeneration after an external perturbation (Lozupone et al., 2012; Lahti et al., 2014; Sommer et al., 2017). The single oral inoculation of healthy adult laying hens in our experiment may therefore have been insufficient to result in a perturbation that could cause a detectable shift in the established gut microbiome of the hens.

Previous studies have also described that colonization after FMT is more successful for genera which were already present in the recipient before FMT, and that rare genera are less likely to colonize (Li et al., 2016; Schmidt et al., 2018). This may explain why the genus *Megamonas* (phylum *Firmicutes*), which was found with a high relative abundance in the duck inoculum, was not detected in any of the hen samples, even though *Megamonas* has been reported to be present in the cecum and feces of laying hens (Videnska et al., 2014a; Polansky et al., 2016). Clearly, *Megamonas* can inhabit the chicken gut, but as it was also absent in the auto inoculum, the gut conditions in the chickens of this study may not have been favorable for *Megamonas*.

We collected cloacal swabs from the chickens because our daily sampling scheme and longitudinal follow-up of the same individual laying hens required a rapid and accurate sampling methodology, without sacrificing the birds. The cloacal swabs were inserted deeply into the cloacal opening to enter the last part of the colon and to ensure the cloacal swabs contained enough fecal material for DNA extraction, we visually assessed the swabs prior to DNA extraction. It has been found that fecal microbiota of chickens were qualitatively similar to the cecal microbiota, but that they differed quantitatively (Stanley et al., 2015) and that the fecal microbiome is more variable than the cecal microbiome (Oakley and Kogut, 2016). Collection of cecal droppings might have therefore been preferable over collection of cloacal swabs but was not feasible in our experimental

design. However, we anticipated that major shifts would have also been picked up by sampling of the fecal microbiome, which has been demonstrated before (Oakley and Kogut, 2016, Jurburg et al., 2019).

Furthermore, it has been proposed that to accurately determine the fate of donor microbiota after FMT, it is necessary to track the microbiota at the resolution of strains rather than at the level of genera or species as is done with 16S rRNA gene amplicon sequencing (Li et al., 2016; Schmidt et al., 2018). For example, Li et al. (2016) demonstrated that single nucleotide variant analysis was able to detect donor strains colonizing the recipient after FMT, where 16S ribosomal RNA gene-based profiling was not sensitive enough to distinguish colonization of donor species from the temporal fluctuations of new species in the recipient. Therefore, it might be possible that certain strains of microbiota were transmitted with the inoculation, but not detected with our method of analysis. Also, we chose to analyze samples of 2 D after inoculation and not to analyze samples collected 1 D after inoculation. This was decided because we expected that samples collected 1 D after inoculation would detect the inoculum after passing through the intestinal tract rather than shifts in the fecal microbiome composition. However, we cannot rule out that minor changes in the fecal microbiome due to inoculation had occurred before 2 D after inoculation.

In conclusion, our findings show that a single oral inoculation of adult laying hens with duck feces in an experimental set-up results in limited effects at the genus level in the gut microbiome of the hens. We detected an increase of *Alistipes* across all treatments, but this may have been an effect of intrinsic temporal fluctuation or of the inoculation procedure itself and could not be attributed to the inoculation with duck feces. Further studies are needed to determine whether repeated exposure of adult chickens to duck feces, which are common in the field, may result in different outcomes, or whether other proxies can be identified that could serve as a measure for contact between ducks and laying hens.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Figure S1. Relative abundance (%) of the 15 most abundant genera displayed per treatment group and faceted per timepoint. Average values per inoculum, and per treatment and timepoint are displayed. Inochicken: chicken inoculum (n = 5). Inoduck: duck inoculum (n = 5). Control: no treatment (n = 7). Auto: inoculation with own chicken feces (n = 7). Duck: inoculation with duck feces (n = 7).

Table S1. Viability counts performed on inocula batches immediately after processing in the lab. The inocula batches were plated on blood agar plates in a fivefold dilution series. Colony forming units (CFU) were counted after overnight incubation at 35°C and CFU/ml were calculated. CFU counts are shown of chicken and duck inocula batches that were used for inoculation of the laying hens on day 0. Prior to inoculation the duck batches were homogenized to form one single duck inoculum.

Table S2. PERMANOVA-like Adonis on Bray-Curtis distances. The treatment groups were tested for significant differences on Bray-Curtis distances between groups per timepoint and within a group between different timepoints.

Table S3. Differences in the relative abundances (%) on selected genera that had a significant difference (Kruskal-Wallis) in relative abundance between the treatments at day 2 or day 7. Inoculum Duck/Auto indicates if the genera were present in the duck (D) or auto (A) inoculum.

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