GENETICS AND GENOMICS

Gastrointestinal microbial population of turkey (*Meleagris gallopavo*) affected by hemorrhagic enteritis virus

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ABSTRACT Hemorrhagic enteritis (HE) is an acute viral disease that affects avian species, particularly turkeys, compromising their commercial production and having a negative effect on animal welfare. Turkey adenovirus 3 (TAdV-3), is the main causal agent of the disease. In this study, we considered 3 groups of turkeys to achieve 2 purposes: 1) A preliminary investigation on the microbiota content in the 4 parts of healthy turkey's intestine (group A), namely duodenum, jejunum, ileum, and ceca was done; 2) an investigation on the relationship between natural infections with TAdV-3 and the intestinal microbiota in the jejunum, where HE mostly develops, comparing group A with animals with molecular positivity for the virus and with clinical signs of HE (group B) and animals with molecular positivity for the virus but without clinical signs (group C). Massive sequencing of the hypervariable V1–V2 regions of 16S rRNA gene and QIIME 1.9.1 software analysis was performed, and operation taxonomic units (OTUs) were classified into 4 abundant phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. The microbial population of small intestine was distributed almost homogeneously in the healthy turkeys, and Firmicutes was the prevalent phylum (79.85% in duodenum, 89.57% in jejunum and 99.28% in ileum). As compared with small intestine, ceca microbial community was much more heterogeneous: Firmicutes (48.03%), Bacteroidetes (33.60%) and Proteobacteria (12.32%). In the natural infections of HEV, the main bacterial families were Bacteroidaceae (Bacteroidetes) and Peptostreptococcaceae (Firmicutes), uniquely detected in group B and C. Also Clostridiaceae (Firmicutes) was detected, uniquely in group B.

Key words: Gut microbiota, hemorrhagic enteritis, turkey, 16S

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INTRODUCTION

The gastrointestinal tract is colonized by beneficial bacteria that are essential for promoting normal intestinal development, physiology, and digestion, establishing meanwhile a mutualistic relationship with the host (Nicholson et al., 2012; Tremaroli and Bäckhed, 2012). Microbial communities have also adapted to colonize different locations in the intestine, allowing unique interactions with the immune system and collectively influencing its intestinal immune cell homeostasis (Cebra, 1999). Dysregulated localization of mutualistic bacteria and dysbiosis is associated with infectious and inflammatory diseases and can lead to inappropriate activation of the immune system (Ma et al., 2011; Oakley et al., 2014; Perumbakkam et al., 2014; Karst, 2016). In avian species, the gastrointestinal tract microbiota is composed by fungi, protozoa and bacteria, the last being the predominant microorganisms. The populations of bacteria keep changing during growth as related to age, diet, breed, and geographic location (Pan and Yu, 2013; Oakley et al., 2014). Avian microbiota is composed at phylum level by Firmicutes, Bacteroidetes and Proteobacteria: *Lactobacillus (*Firmicutes*)*, *Bacilli (*Firmicutes*)* and *Enterococcus (*Firmicutes*)* are the most abundant genera (Wei et al., 2013; Mancabelli et al., 2016). In commercially important species such as chickens and turkeys, the microbiota has been recently reviewed (Pan and Yu, 2013; Waite and Taylor, 2015) and a phylogenetic diversity census of poultry intestinal bacteria showed that chickens and turkeys share only 16% similarity between their respective microbiotas at the species-equivalent level (Wei et al., 2013).

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Ethical interest: Ethical standards for commercial turkey production were followed by the company during fattening and slaughtering of the animals.

Information about the microbiota composition, using the 16S rRNA gene of the regions of small intestine in turkey species such as duodenum and jejunum, we found that an investigation was carried out to identify the microbiota of heavy and light turkey flock, in order to study the relationship between bacterial community composition and the insurgency of Light Turkey Syndrome (Danzeisen et al., 2013). However, study of the microbiota composition of the regions of small intestine in healthy or in pathological conditions is lacking. Instead, a more recent study investigated the temporal relationship between ileal microbiota during development, and the impact of low-dose penicillin on bacterial community of ileum and cecum (Danzeisen et al., 2015). At the ceca level, differences in the bacteria genera present in turkeys were determined (Scupham et al., 2008), and time-dependent differences in turkey intestinal tract were identified as well (Scupham, 2009) by means of culture-based or lower-output molecular fingerprint methods, such as automated rRNA intergenic spacer analysis (**ARISA**) or terminal restriction fragment length polymorphism (**T-RFLP**) analysis. The methods used had limitations and biases that could be exceeded with the advent of next-generation sequencing (**NGS**). It has fueled the metagenomics studies to identify the genomes of entire communities, including those of uncultivated organisms, using the 16S rRNA gene because it is a marker for investigating bacterial phylogeny (Tremblay et al., 2015).

Hemorrhagic enteritis (**HE**) is an acute viral disease affecting mainly turkeys (*Meleagris gallopavo*) 4 wk of age and older caused by turkey adenovirus 3 (TAdV-3), member of genus *Siadevovirus* (Sharma, 1991; Beach et al., 2009). The common clinical signs of the disease include depression, bloody droppings, and death; it is likely transmitted through the fecal-oral/cloacal route (Dhama et al., 2017). Oral infection of susceptible turkeys with pathogenic TAdV-3 strains results in well-characterized splenomegaly and intestinal bleeding in 4 to 6 d, causing subclinical infections and mortality (Suresh and Sharma, 1996). Hemorrhagic adenovirus is one of the most important causes of economic loss to the turkey industry; mortality ranges between 10 to 15% but can reach 60% in some flocks (Dhama et al., 2017). Infection with HE virus (**HEV**) results in a transient immunosuppression, paving the ways for other diseases that can strike the animals that survived the first wave of infection. This second wave of infections is lethal for the majority of animals, given that colisepticemia often follows clinical and subclinical infections with HE 12 to 14 d later (Moura-Alvarez et al., 2014). Gross pathology presents dilated intestine with blood content and a yellowish substance on the intestinal mucosa (Dhama et al., 2017). At the microscopic level, severe congestion in the intestinal mucosa, degeneration and shortening of the villi, and bleeding at the tips of the villi are observed (Sharma, 1991). Given the background that viruses infecting the gastrointestinal tract are related to the host microbiota and that emerging data suggest enteric viruses are regulated by microbial population through a series of processes termed "trans-kingdom interactions" (Pfeiffer and Virgin, 2016), the aim of the present investigation was to characterize the relationship between HEV infection and the turkey intestinal microbiota by means of NGS of 16S rRNA genes. The first part of this study analyzed the microbiota of the 4 intestinal tracts, including duodenum, jejunum, ileum, and cecum, collected from healthy turkeys. The second part aims to understand the changes in the jejunum microbiota in HEV-infected turkeys, by comparing HEV-positive animals, either with or without clinical signs, to healthy ones.

MATERIALS AND METHODS

Sample Collection

The present study was carried out on commercial B.U.T. BIG6 hybrid turkeys. Ethical standards for commercial turkey production were therefore followed by the company during fattening and slaughtering of turkeys. All the samples were collected from prepuberal females 80 d of age.

The microbiota from intestinal tracts was determined from 3 groups of turkeys, including healthy turkeys (group A), HE-affected turkeys (group B), and turkeys positive for HEV but without clinical signs (group C). Four clinically healthy turkeys were sorted out during routine slaughtering procedures (group A). Pathological analysis of the gastroenteric tract evidenced no sign of gross pathological lesions related to enteritis. Molecular diagnosis to rule out the presence of HEV was carried out in the 4 tracts of intestine and spleen by means of specific PCR (Hess et al., 1999) confirming that the animals were healthy and no virus was present in their organism. Intestinal content was collected from 2 cm tract of each district, including jejunum, duodenum, ileum, and ceca, by scraping the intestinal mucosa with a sterile plastic scraper (Cell Scrapers, Sterile, Greiner Bio-One, VWR, Milano, Italy). Collected samples were snap frozen in liquid nitrogen.

A second set of samples (group B) was collected from a group of 4 turkeys with evident acute clinical signs of HE. The animals were subjected to euthanasia by cervical dislocation due to their critical clinical condition. Gross pathology confirmed the presence of enteritis lesions compatible with acute HE infection in jejunum, but no signs were found on the other districts of the intestine. The presence of HEV was confirmed with molecular diagnosis via polymerase chain reaction (**PCR**), which was positive in both jejunum and spleen. Samples for microbiota determination were collected as previously described for healthy animals.

A third set of 4 samples (group C) was collected during routine slaughtering procedures from animals that did not evidence any clinical sign of HE although being raised in the same barn of the infected turkeys. Gross pathology did not evidence any lesion throughout

intestinal tracts. Molecular diagnosis of intestine sections was negative. On the contrary, presence of HEV was confirmed in spleen by PCR.

All animals included in the experiment were never treated with antibiotics or probiotics.

Bacterial DNA Extraction and Sequence Analysis

The bacterial DNA was extracted using Powersoil® DNA extraction kit (Mobio), according to the manufacturer's instructions. DNA samples were eluted in 100 μL and stored at –20°C until further processing. The DNA concentration was quantified by NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies) and A_{260}/A_{280} ratio was ~1.8. The bacterial hypervariable regions V1-V2 of 16S rRNA gene were amplified by PCR with primer pair F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R338 (5′-TGCTGCCTCCCGTAGGAGT-3′). Both primers included sequencing adaptors at the 5′ end and forward primers were tagged with different barcodes. These hypervariable regions were chosen by the frequency with which they are used in research and because they contain a high discriminatory power for bacterial species (Chakravorty et al., 2007; Engelbrektson et al., 2010; Fouhy et al., 2016). PCR mixture (50 μL) contained 2 μL of DNA template (~5 ng), 5 μL of 10× AccuPrime™ PCR Buffer II, 0.2 μM of each primer and 1 U of AccuPrime™ Taq DNA Polymerase High Fidelity (Life Technologies). The PCR thermal profile was 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final extension of 7 min at 72°C. For each amplicon, both concentration and quality were determined using Agilent Bioanalyzer 2100. Samples were sequenced on an Ion Torrent Personal Genome Machine (**PGM**) with the Ion 318 Chip Kit v2 (Life Technologies) under manufacturer's conditions.

Bioinformatics and Data Analysis

Raw reads (https://www.ncbi.nlm.nih.gov, BioProject PRJNA347549) were de-multiplexed, quality-filtered and analyzed using QIIME 1.9.1 software (Caporaso et al., 2010). Reads included had a length greater than 300 nt; a mean quality score above 25 in sliding window of 50 nucleotides; no mismatches on the primer; and default values for other quality parameters.

Quality-filtered reads were clustered into operational taxonomic units (**OTUs**) at 97% similarity, using UCLUST in an open reference approach for taxonomy analyses. Taxonomic assignment of representative OTUs was performed using the RDP Classifier (Wang et al., 2007) against Greengenes v13.8 database. Alignment of sequences was performed using PyNast (Caporaso et al., 2010) as default in QIIME pipeline. Via VSEARCH (Westcott and Schloss, 2015; Rognes et al., 2016), the chimeric sequences (24.1%) were removed, and then we applied 2 filtering steps in aligned and taxonomy-assigned OTU table: first, sequences corresponding to chloroplast class were filtered out, and then sequences that represent less than 0.005% of total OTUs were also filtered out from the OTU table. Downstream analyses were performed using QIIME 1.9.1 (Caporaso et al., 2010) at a depth of 78,500 sequences per sample for the healthy group and 93,300 sequences for the comparison of the jejunum tract for the 3 groups of turkeys to standardize for unequal sequencing depth of the samples. In alpha diversity (within a sample) we used 2 different metrics: observed species (that considers only the richness of OTUs) and Shannon index (that estimates the relative abundance of OTUs in addition to the richness). In beta diversity (between samples), Unweighted UniFrac distance matrix (Lozupone et al., 2011) was used to create PCoA plots. Adonis and ANOSIM were used to assess the statistical differences among the 4 intestinal tracts of the healthy turkeys, and among the 3 jejunum groups corresponding to different health status of turkeys.

RESULTS

To assess composition of intestinal turkeys' microbiota in healthy and HEV-infected birds, we analyzed 3 groups of animals (each group comprised of 4 animals): healthy (group A), HE affected (group B), and HEV-positive but without clinical signs (group C).

We investigated on 1) the composition of intestinal microbiota in 4 healthy turkeys (group A), collecting a total 16 samples, from the fourth intestinal tract: 4 duodenum, 4 jejunum, 4 ileum and 4 ceca samples. Then 2) we compared the 4 jejunum samples of group A, with 8 jejunum samples of turkeys belonging to 2 different group, depending on the health status: 4 HE-affected turkeys (group B) and 4 HEV-positive turkeys without clinical signs (group C).

Group A: Intestinal Microbiota of Healthy Turkeys

A total of 3,400,569 reads were obtained and divided in 654 OTUs. Firmicutes is the most abundant phylum, almost 80% in duodenum, then values rising almost to 90% in jejunum and to 98% in ileum. The abundance of Firmicutes diminishes in the ceca (46.44%). At the duodenum taxonomic level, the most abundant family from Firmicutes is Lactobacillaceae (72.45%). The remainder OTUs belonged to Proteobacteria (6.75%), Actinobacteria (3.65%), Bacteroidetes (3.22%) and Cyanobacteria (0.56%). In jejunum, with 87.82% of OTUs corresponding to Firmicutes, Lactobacillaceae provides the 86.47% of the sequences at family level. In ileum, Firmicutes represented the 97.94% of bacteria detected, and the main families are Lactobacillaceae (52.82%) and Clostridiaceae (42.93%). Comparing with duodenum and jejunum, in the ileum we observed a drop in

Table 1. Main bacterial phyla and families obtained for intestine tracts in healthy turkeys.

	Duodenum	Jejunum	Ileum	Cecum
ACTINOBACTERIA	**3.65%**	**4.01%**	**0.00%**	**0.02%**
Micrococcaceae	*1.01%*	*2.56%*	*0.00%*	*0.01%*
Propionibacteriaceae	*2.64%*	*1.45%*	*0.00%*	*0.01%*
BACTEROIDETES	**3.22%**	**0.28%**	**0.10%**	**31.32%**
Bacteroidaceae	*1.87%*	*0.05%*	*0.06%*	*17.87%*
[Barnesiellaceae]	*0.08%*	*0.00%*	*0.01%*	*2.26%*
Prevotellaceae	*0.49%*	*0.11%*	*0.01%*	*3.31%*
Others < 0.99%	*0.77%*	*0.11%*	*0.02%*	*7.89%*
CYANOBACTERIA	**0.56%**	**0.08%**	**0.02%**	**3.89%**
Others < 0.99%	*0.56%*	*0.08%*	*0.02%*	*3.89%*
FIRMICUTES	**76.39%**	**87.82%**	**97.94%**	**46.44%**
Clostridiaceae	*0.30%*	*0.30%*	*42.93%*	*1.85%*
Lactobacillaceae	*72.45%*	*86.47%*	*52.82%*	*1.98%*
Lachnospiraceae	*1.91%*	*0.20%*	*0.07%*	*15.35%*
Ruminococcaceae	*0.20%*	*0.11%*	*0.01%*	*2.31%*
Turicibacteraceae	*0.00%*	*0.01%*	*2.01%*	*0.12%*
Veillonellaceae	*0.60%*	*0.20%*	*0.07%*	*16.66%*
Others < 0.99%	*0.93%*	*0.53%*	*0.04%*	*8.16%*
PROTEOBACTERIA	**6.75%**	**0.30%**	**0.08%**	**11.51%**
Alcaligenaceae	*1.07%*	*0.17%*	*0.04%*	*11.43%*
Campylobacteraceae	*1.79%*	*0.04%*	*0.04%*	*0.07%*
Comamonadaceae	*2.27%*	*0.03%*	*0.00%*	*0.01%*
Neisseriaceae	*1.62%*	*0.06%*	*0.00%*	*0.01%*
SYNERGISTETES	**0.01%**	**0.00%**	**0.01%**	**1.38%**
Others < 0.99%	*0.01%*	*0.00%*	*0.01%*	*1.38%*

Bacterial phyla (italic) and families (italic) that showed a percentage higher than 0.99% at least in one tract of the intestine were represented. Bacterial families that showed a percentage lower than 0.99% were gathered in the "*Others < 0.99%*" group.

the presence of Lactobacillaceae family and the appearance of OTUs from the Clostridiaceae family, which increases from 0.30% in the duodenum and 0.30% in the jejunum to 42.93% in the ileum (Table 1, Figure 1 and, in explanation to Figure 1, the supplementary Table S1). The present results mostly agree with those previously reported (Danzeisen et al., 2013, 2015), which identified Lactobacillaceae and Clostridiaceae as the most abundant families in the ileum and ceca respectively, in a commercial flock of turkeys (62% and 36% respectively).

The cecum shows more diversity at the phylum level, with Firmicutes (46.44%), Bacteroidetes (31.32%) and Proteobacteria (11.51%) being the most abundant phyla, whereas at family level there are more bacterial communities as compared to the other tracts. The most abundant families are Lachnospiraceae (15.35%) and Veillonellaceae (16.66%) for Firmicutes; Bacteroidaceae (17.87%; Bacteroidetes), and Alcaligenaceae (11.43%; Proteobacteria) (Table 1, Figure 1 and, in explanation to Figure 1, the supplementary Table S1). Although the present results are consistent to those previously reported in a meta-analytic investigation (Wei et al., 2013), they differ from others previously reported (Danzeisen et al., 2015), which demonstrated the presence of a higher concentration of clostridia species in ceca (more than 70% of the bacterial population).

Differences in bacterial communities among the 4 intestinal parts of healthy turkeys (duodenum, jejunum, ileum and cecum) were analyzed using the rarefaction curves, PCoA plots and phylogeny-based unweighted UniFrac distance matrix, obtained with QIIME pipeline. Rarefaction curves were generated with a depth of 78,500 for richness (observed species) and evenness (Shannon index) to describe the alpha diversity. Values of the Observed species and Shannon index in the different zone of the intestine in healthy turkeys are showed in Table 2. The species richness from the ceca tract is higher than the other tracts.

Beta diversity was calculated by unweighted UniFrac phylogenetic distance matrix. The PCoA plot in Figure 2 shows a clustering of samples for ceca. For statistical testing, we applied analysis of similarity (**ANOSIM**) and Adonis tests to determine significant differences considering a probability (P-value) less than 0.05 to denote significance in microbial communities among these intestine tracts. Adonis test shows a P-value of 0.001 and the R2 value (effect size), which shows a percentage of variation of 0.43. This means that clustering samples among different tracts of intestine explained 43% of the distance among samples. The same was done for the ANOSIM test: obtaining a P-value of 0.002 and an R2 value of 0.52, it indicates dissimilarity between the intestinal tracts; it is explicated by the changes of bacterial abundance in the alpha diversity analysis (Table 2).

Group A – Group B – Group C: Differences in Jejunum Tract during HEV Infection

A total of 2,199,136 reads were obtained and divided in 654 OTUs. The second part of this study explored the relationship between natural HEV infection and the microbial community. Focus was on the jejunum, which is the region of the gastrointestinal apparatus that is more affected by HEV infection. Microbial

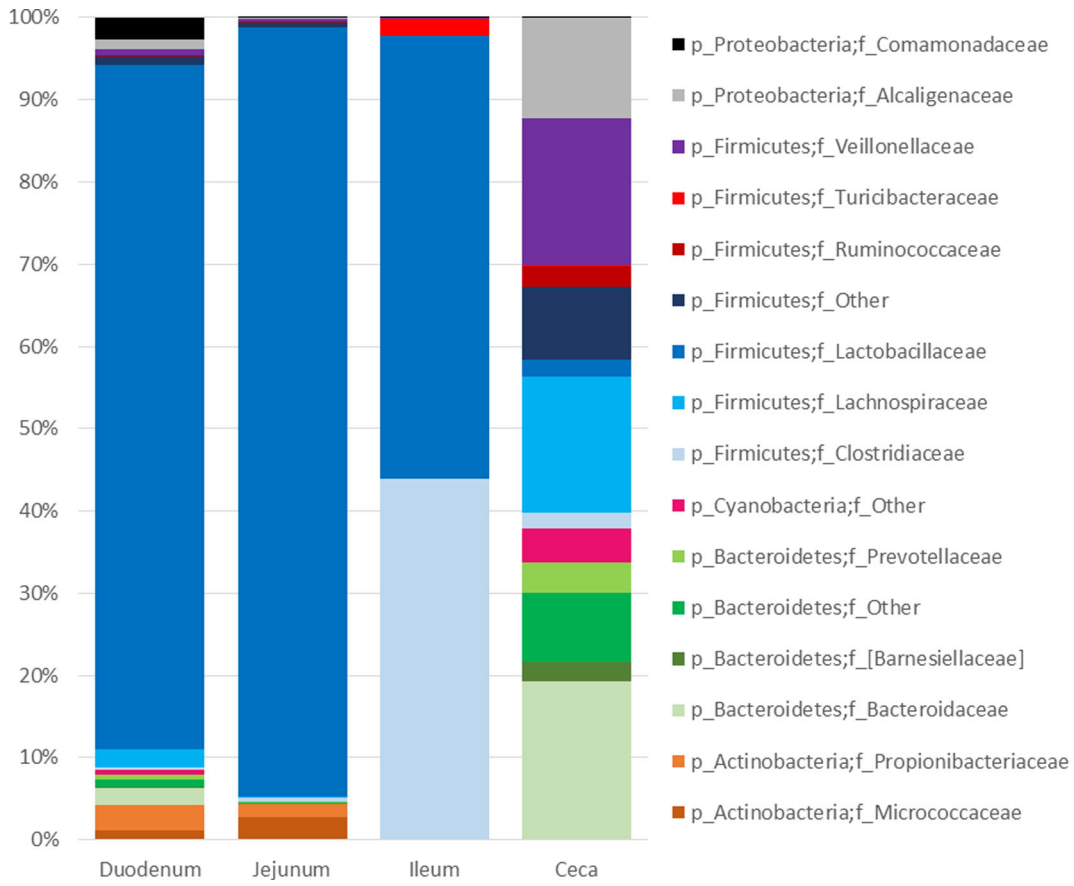

Figure 1. Main bacterial families and corresponding phyla, in the 4 tracts of the intestine in the healthy turkeys: duodenum, jejunum, ileum and cecum. At family level, "Other" means that level that have a percentage too low for assigning the taxonomy. Percentage details are showed in Table 1 and supplementary Table S1.

Table 2. Values of Observed species and Shannon index in different tract of the intestine in healthy turkeys.

SHANNON	MEAN	STD
Duodenum	4.83	0.61
Jejunum	4.29	0.54
Ileum	4	0.55
Cecum	5.71	0.57
OBSERVED SPECIES	MEAN	STD
Duodenum	233	28.24
Jejunum	199.25	31.11
Ileum	255.75	33.01
Cecum	390.5	25

community was collected from healthy turkeys (group A), HE-affected turkeys (group B), and turkeys positive for HEV but without clinical signs (group C). The most striking difference was the evident decrease of the Lactobacillaceae in HE-affected animals (65.16%) as compared to the 86.47% of the healthy animals. Clostridiaceae had a higher percentage in HE-affected animals (7.35%), compared with the other 2 groups of animals (<0.3%) (Table 3, Figure 3 and, in explanation to Figure 3, the supplementary Table S2).

Interestingly, there are 2 families, Bacteroidaceae and Peptostreptococcaceae, which are detected in jejunum tract of groups B and C, but are absent in group A (Figure 3).

The procedure was the same used for the analysis in the different tracts of the intestine in healthy animals. We obtained rarefaction curves with a depth of 93,300 and the observed species values were: 200.5 in group A; 202.5 in group B; 165 in group C (Table 4). The richness and the evenness of bacterial species is homogeneous in the 3 groups. When we analyze data with the unweighted UniFrac distance matrix (Figure 4), we identified 2 clusters of samples: the group A, is separated from groups B and C but none statistically significant results were found with ANOSIM and Adonis tests.

DISCUSSION

Group A: Intestinal Microbiota of Healthy Turkeys

The distribution of bacterial communities and the richness of bacterial families did not present any statistically significant difference among the healthy animals, probably due to the fact that the animals recruited in the present experiment were reared in the same flock, and homogeneous for feeding and sampling time. Age, sex, genetic background of the host, and diet are regarded as the main factors influencing the

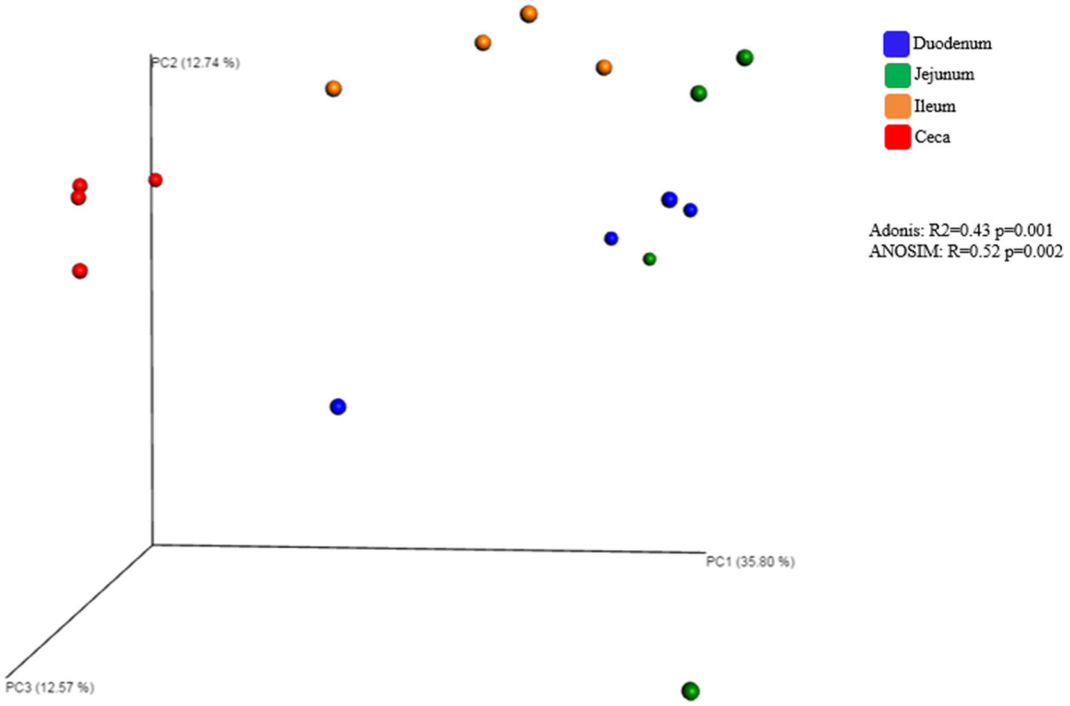

Figure 2. Unweighted UniFrac analysis among the 4 intestine tracts in 4 healthy turkeys (group A). Sample from cecum are clustered separately from the other tracts of the intestine.

Table 3. Comparison of bacterial composition in the jejunum tract in 12 turkeys from 3 different groups. Group (A): 4 healthy animals. Group (B): 4 HEV affected turkeys positive for PCR at intestinal level and with clinical signs and evident gross pathological lesions. Group (C): 4 positive turkeys for HEV PCR at spleen level, but without clinical signs or gross pathological lesions. Bacterial phyla (bold) and families (italic) that showed a percentage higher than 0.99% at least in one tract of the intestine were represented. Bacterial families that showed a percentage lower than 0.99% were gathered in the "*Others < 0.99%*" group.

	Group A	Group B	Group C
ACTINOBACTERIA	**4.01%**	**2.57%**	**0.39%**
Micrococcaceae	*2.56%*	*0.76%*	*0.05%*
Propionibacteriaceae	*1.45%*	*1.81%*	*0.34%*
BACTEROIDETES	**0.05%**	**5.33%**	**3.61%**
Bacteroidaceae	*0.05%*	*5.33%*	*3.61%*
FIRMICUTES	**87.17%**	**81.96%**	**89.95%**
Clostridiaceae	*0.30%*	*7.35%*	*0.09%*
Lactobacillaceae	*86.47%*	*65.16%*	*83.85%*
Lachnospiraceae	*0.20%*	*1.42%*	*0.80%*
Peptostreptococcaceae	*0.00%*	*6.48%*	*4.21%*
Veillonellaceae	*0.20%*	*1.55%*	*1.00%*
PROTEOBACTERIA	**0.04%**	**0.75%**	**1.06%**
Campylobacteraceae	*0.04%*	*0.75%*	*1.06%*

composition of gastrointestinal microbiota in each intestinal tract in both mammalian (Langille and Zaneveld, 2013) and avian species (Wilkinson et al., 2016). Any difference in ceca microbiota related to age can be ruled out, given that the analysis was carried out on animals with an age corresponding to that of previous experiments (Danzeisen et al., 2015). The differences between the present results and those previously reported may be related to genetic differences. The present investigation was carried out on a homogeneous population of hybrid B.U.T. BIG6 turkeys. The previous studies did not provide this information. Different genetic basis may have an impact on immune system, which is known to be related to the microbiota development in the gastrointestinal tract.

A recent study on Japanese quail (*Coturnix japonica*) demonstrated that sex differences can have a major impact on cecum microbiota (Wilkinson et al., 2016). We may also speculate that differences between the present investigation and the other previously reported might also be due to sex differences between the groups of animals included in the respective studies, at least for what concerns the study of Danzeisen et al., (2013), whereas the turkey microbiota of ceca and Ileum (Danzeisen et al., 2015) was determined on an homogeneous female population.

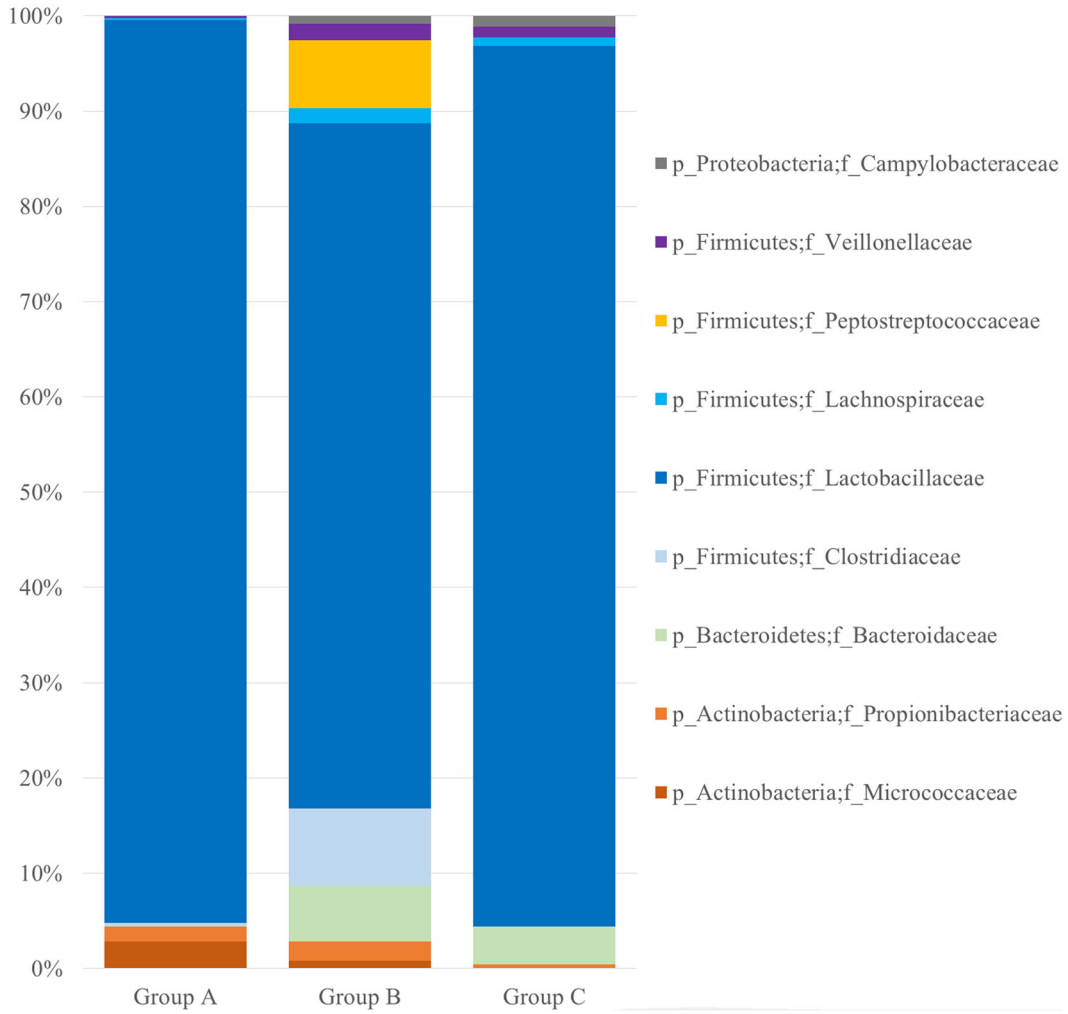

Figure 3. Main bacterial families and corresponding phyla among the 3 groups of turkeys. Healthy turkeys (group A), HEV-affected turkeys (group B) and turkeys positive for HEV but without clinical signs (group C). At family level, "Other" means that level that have a percentage too low for assigning the taxonomy. Percentage details are showed in Table 3 and supplementary Table S2.

Table 4. Values of Observed species and Shannon index among 3 group of turkeys in 3 different healthy status.

SHANNON	MEAN	STD
Group A	4.29	0.53
Group B	4.07	0.58
Group C	3.25	1.16
OBSERVED SPECIES	MEAN	STD
Group A	200.5	33.55
Group B	202.5	43.04
Group C	165	24.5

We compare the jejunum tract of the 3 group. Group A: healthy turkeys, Group B: HEV affected turkeys, Group C: turkeys positive for HEV but without clinical signs

The microbial distribution agrees the result reported in a study on chicken, which demonstrated that the community of small intestine, namely duodenum, jejunum, and ileum, is more homogeneous than the microbial community of ceca (Oakley et al., 2014). The small intestine harbors mostly Lactobacillaceae and Clostridiaceae, as confirmed by the present study, their role being to mediate starch breakdown and lactic acid fermentation. On the contrary, as compared to small intestine, microbial community is much more heterogeneous in ceca, which acts as a large reservoir for the commensal bacteria that are involved in the fermentative digestion of the complex carbohydrates that cannot be dealt with by small intestinal enzymes (Waite and Taylor, 2014).

Group A – Group B – Group C: Differences in Jejunum Tract during HEV Infection

The aim was to determine the relationship between the jejunum microbiota and natural HEV infection, considering turkeys with or without clinical sign and evident gross pathological lesions (group B and C respectively) and compare them with the healthy one (group A) to see if a difference in the distribution of bacterial communities occurs. From a genomic perspective, Peptostreptococci are more closely related to clostridia than Streptococci (Murray et al., 1991). *Peptostreptococcus* species are commensal organisms in chicken ileum (Mohd Shaufi et al., 2015), and their presence have been shown to be modified (albeit reduced) in

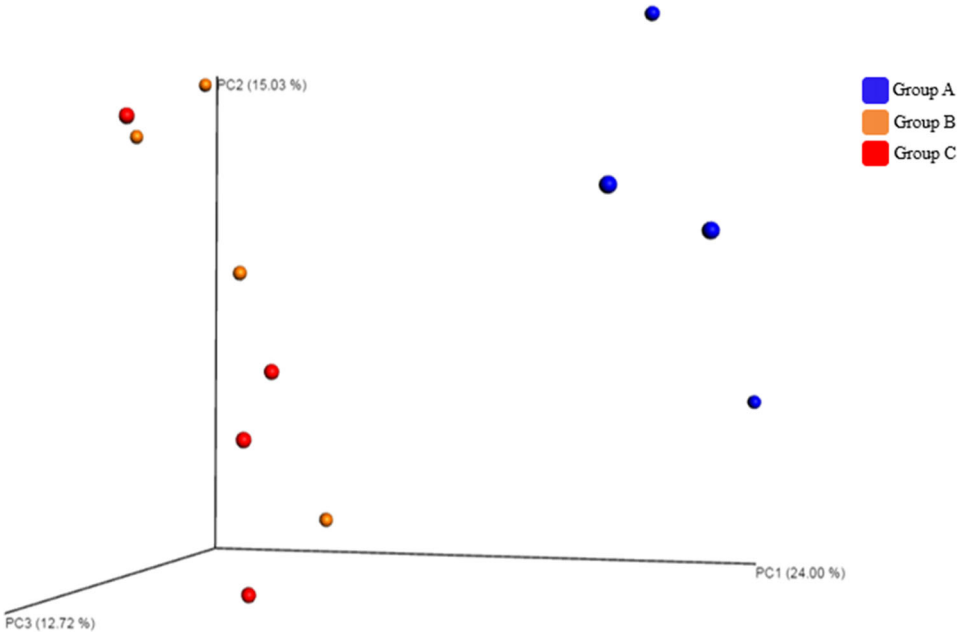

Figure 4. Unweighted UniFrac analysis among the 3 health status of animals; 4 healthy turkeys (group A), 4 HEV-affected turkey (group B) and 4 turkeys positive for HEV but without clinical signs (group C).

faeces after Marek virus infection (Perumbakkam et al., 2014). In turkey, the cecal presence of Peptostreptococci was significantly increased in high fiber-fed turkeys (Bedbury and Duke, 1983). *Bacteroides* spp are anaerobic, non-spore forming, gram-negative rods that are normally found in the lower digestive tract, especially ceca, of poultry. *Bacteroides* are rarely associated with diseases. *Bacteroides fragilis* has been isolated from salpingitis in laying hens (Bisgaard and dam, 1981), and *Bacteroides* has been associated with phallus inflammation of ganders (Behr et al., 1990).

Multiple hypotheses regarding the immunopathogenesis of HE and related viruses have been proposed. Based on the work of Rautenschlein and Sharma (2000) it was suggested the following, editorialized model. After oral exposure, HEV either undergoes an initial round of replication in B-lymphocytes located in the intestine and Bursa of Fabricius, or it travels directly to the spleen via the peripheral blood where it infects more B-cells and macrophages and replicates to high numbers. This results in an influx of CD4+ T-cells and macrophages into the white pulp, presumably in an attempt to clear virus and/or support the immune reaction, and accounts for the spleen hyperplasia observed during the acute phase of infection. We speculate that the animals of group C, on the background that they did not present any pathological nor histopathological lesion in duodenum, were less susceptible to the lesions of the virus, which was still present at spleen level. We cannot rule out the possibility that the different composition of bacterial community lies at the background of this different susceptibility to the intestinal disease.

The results presented in this investigation provides the background for future studies aimed at deciphering host-microbiota and microbe-microbe interactions to improve turkey health through the modulation of microbial intestinal population, providing the knowledge to enhance bird growth and improve turkey immune defences against enteric diseases.

SUPPLEMENTARY DATA

Supplementary data are available at *PSCIEN* online.

Table S1. Complete table of bacteria species found up to Phylum level (L2), Class level (L3), Order level (L4), Family level (5) and Genus level (L6), in the 4 tracts of the intestine. The value 0.00% indicates a percentage lower than 0.0005%. "Other": Class, Order, Family or Genus level that have a percentage too low for assigning the taxonomy. "N.C.": bacteria that can't be classified until corresponding Class, Order, Family or Genus level.

Table S2. Complete table of bacteria species found up to Phylum level (L2), Class level (L3), Order level (L4), Family level (5) and Genus level (L6). Group A: healthy turkeys, Group B: turkeys with clinical signs of HEV, Group C: positive turkeys to HEV but without clinical signs. The value 0.00% indicates a percentage lower than 0.0005%. "Other": Class, Order, Family or Genus level that have a percentage too low for assigning the taxonomy. "N.C.": bacteria that can't be classified until corresponding Class, Order, Family or Genus level.

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REFERENCES

Beach, N. M., R. B. Duncan, C. T. Larsen, X. J. Meng, N. Srianganathan, and W. F. Pierson. 2009. Comparison of 12 turkey hemorrhagic enteritis virus isolates allows prediction of genetic factors affecting virulence. J. Gen. Virol. 90:1978–1985.

Bedbury, H. P., and G. E. Duke. 1983. Cecal microflora of turkeys fed low or high fiber diets: enumeration, identification, and determination of cellulolytic activity. Poult. Sci. 62:675–682.

Behr, K. P., K. H. Hinz, and S Rottmann. 1990. Phallus-inflammation of ganders: clinical observations and comparative bacteriological examinations of healthy and altered organs. Zentralbl. Veterinarmed. B. 37:774–776.

Bisgaard, M., and A Dam. 1981. Salpingitis in poultry. II. Prevalence, bacteriology, and possible pathogenesis in egg-laying chickens. Nord. Vet. Med. 33:81–89.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, E. Jeremy, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7:335–336.

Cebra, J. J. 1999. Influences of microbiota on intestinal immune system. 69:1046–1051.

Chakravorty, S., D. Helb, M. Burday, and N. Connell. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods. 69:330–339.

Danzeisen, J. L., A. J. Calvert, S. L. Noll, B. McComb, J. S. Sherwood, C. M. Logue, and T. J. Johnson. 2013. Succession of the turkey gastrointestinal bacterial microbiome related to weight gain. PeerJ. 1:e237.

Danzeisen, J. L., J. B. Clayton, H. Huang, D. Knights, B. McComb, S. S. Hayer, and T. J. Johnson. 2015. Temporal relationships exist between cecum, ileum, and litter bacterial microbiomes in a commercial turkey flock, and subtherapeutic penicillin treatment impacts ileum bacterial community establishment. Front. Vet. Sci. 2:56.

Dhama, K., V. Gowthaman, K. Karthik, R. Tiwari, S. Sachan, M. A. Kumar, M. Palanivelu, Y. S. Malik, R. K. Singh, and M. Munir. 2017. Haemorrhagic enteritis of turkeys – current knowledge. Vet. Q. 37:31–42.

Engelbrektson, A., V. Kunin, K. C. Wrighton, N. Zvenigorodsky, F. Chen, H. Ochman, and P. Hugenholtz. 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J. 4:642–647.

Fouhy, F., A. G. Clooney, C. Stanton, M. J. Claesson, and P. D. Cotter. 2016. 16S rRNA gene sequencing of mock microbial populations- impact of DNA extraction method, primer choice and sequencing platform. BMC Microbiol. 16:123. doi:10.1186/s12866-016-0738-z.

Hess, M., R. Raue, and H. M. Hafez. 1999. PCR for specific detection of haemorrhagic enteritis virus of turkeys, an avian adenovirus. J. Virol. Methods. 81:199–203.

Karst, S. M. 2016. The influence of commensal bacteria on infection with enteric viruses. Nat. Rev. Microbiol. 14:197–204.

Langille, M. G. I., and J. Zaneveld. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31:1–20.

Lozupone, C., M. E. Lladser, D. Knights, J. Stombaugh, and R. Knight. 2011. UniFrac: An effective distance metric for microbial community comparison. ISME J. 5:169–172.

Ma, C., X. Wu, M. Nawaz, J. Li, P. Yu, J. E. Moore, and J. Xu. 2011. Molecular characterization of fecal microbiota in patients with viral diarrhea. Curr. Microbiol. 63:259–266.

Mancabelli, L., C. Ferrario, C. Milani, M. Mangifesta, F. Turroni, S. Duranti, G. A. Lugli, A. Viappiani, M. C. Ossiprandi, D. van Sinderen, and M. Ventura. 2016. Insights into the biodiversity of the gut microbiota of broiler chickens. Environ. Microbiol. 18:1462–2912.

Mohd Shaufi, M. A., C. C. Sieo, C. W. Chong, H. M. Gan, and Y. W. Ho. 2015. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut Pathog. 7:4.

Moura-Alvarez, J., L. F. N. Nuñez, C. S. Astolfi-Ferreira, T. Knöbl, J. L. Chacón, A. M. Moreno, R. C. Jones, and A. J. P. Ferreira. 2014. Detection of enteric pathogens in Turkey flocks affected with severe enteritis, in Brazil. Trop. Anim. Health Prod. 46:1051–1058.

Murray, B. E., K. V. Singh, S. M. Markowitz, H. A. Lopardo, J. E. Patterson, M. J. Zervos, E. Rubeglio, G. M. Eliopoulos, L. B. Rice, F. W. Goldstein, S. G. Jenkins, G. M. Caputo, R. Nasnas, L. S. Moore, E. S. Wong, and G. Weinstock (1991). Evidence for clonal spread of a single strain of β-lactamase-producing *Enterococcus* (*Streptococcus*) faecalis to six hospitals in five states. J. Infect. Dis. 163:780–785.

Nicholson, J. K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, and S. Pettersson. 2012. Host-gut microbiota metabolic interactions. Science. 336:1262–1267.

Oakley, B. B., H. S. Lillehoj, M. H. Kogut, W. K. Kim, J. J. Maurer, A. Pedroso, M. D. Lee, S. R. Collett, T. J. Johnson, and N. A. Cox. 2014. The chicken gastrointestinal microbiome. FEMS Microbiol. Lett. 360:100–112.

Pan, D., and Z. Yu. 2013. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108–119.

Perumbakkam, S., H. D. Hunt, and H. H. Cheng. 2014. Marek's disease virus influences the core gut microbiome of the chicken during the early and late phases of viral replication. FEMS Microbiol. Ecol. 90:300–312.

Pfeiffer, J. K., and H. W. Virgin. 2016. Transkingdom control of viral infection and immunity in the mammalian intestine. Science. 351:aad5872.

Rautenschlein, S, and J. M. Sharma. 2000. Immunopathogenesis of haemorrhagic enteritis virus (HEV) in turkeys. Dev. Comp. Immunol. 24:237–246.

Rognes, T., T. Flouri, B. Nichols, C. Quince, and F. Mahé. 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ Prepr. 4:e2409v1.

Scupham, A. J. 2009. Campylobacter colonization of the turkey intestine in the context of microbial community development. Appl. Environ. Microbiol. 75:3564–3571.

Scupham, A. J., T. G. Patton, E. Bent, and D. O. Bayles. 2008. Comparison of the cecal microbiota of domestic and wild turkeys. Microb. Ecol. 56:322–331.

Sharma, J. M. 1991. Hemorrhagic enteritis of turkeys. Vet. Immunol. Immunopathol. 30:67–71.

Suresh, M., and J. M. Sharma. 1996. Pathogenesis of type II avian adenovirus infection in turkeys: in vivo immune cell tropism and tissue distribution of the virus. J. Virol. 70:30–36.

Tremaroli, V., and F. Bäckhed. 2012. Functional interactions between the gut microbiota and host metabolism. Nature. 489:242–249.

Tremblay, J., K. Singh, A. Fern, E. S. Kirton, S. He, T. Woyke, J. Lee, F. Chen, J. L. Dangl, and S. G. Tringe. 2015. Primer and platform effects on 16S rRNA tag sequencing. Front. Microbiol. 6:771.

Waite, D. W., and M. W. Taylor. 2014. Characterizing the avian gut microbiota: Membership, driving influences, and potential function. Front. Microbiol. 5:223.

Waite, D. W., and M. W. Taylor. 2015. Exploring the avian gut microbiota: current trends and future directions. Front. Microbiol. 6:673.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73:5261–5267.

Wei, S., M. Morrison, and Z. Yu. 2013. Bacterial census of poultry intestinal microbiome. Poult. Sci. 92:671–683.

Westcott, S. L., and P. D. Schloss. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. PeerJ. 3: e1487.

Wilkinson, N., R. J. Hughes, W. J. Aspden, J. Chapman, R. J. Moore, and D. Stanley. 2016. The gastrointestinal tract microbiota of the Japanese quail, *Coturnix japonica*. Appl. Microbiol. Biotechnol. 100:4201–4209.