Use of 16S rRNA gene sequencing for prediction of new opportunistic pathogens in chicken ileal and cecal microbiota

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ABSTRACT In this study, we addressed differences in the development of gut microbiota in 4 successive batches of commercially hatched broiler parent chickens. When planning this study, we expected to find a batch with compromised performance which would allow identification of microbiota of suboptimal composition. Microbiota composition was determined only by sequencing the V3/V4 region of 16S rRNA genes in samples collected from chickens 5 to 18 wk of age. In a total, 100 and 160 samples originating from the ileum or cecum were processed, respectively. In one of the flocks with suboptimal performance we identified an increased abundance of *Helicobacter brantae* forming over 80% of ileal microbiota in individual chickens. Moreover, we also tested samples of 53-wk-old hens from the same genetic line in which egg production decreased. In this case, cecal microbiota was enriched for *Fusobacterium mortiferum* forming over 30% of total cecal microbiota. Although none of the identified unusual microbiota members have been well recognized as pathogenic, they may represent new opportunistic pathogens of chickens worth of further investigation. Analysis of gut microbiota composition by next generation sequencing thus proved as a useful and unbiased alternative to bacterial culture, especially in the cases of unspecific symptoms like decrease in flock performance.

Key words: chicken, microbiota, broiler parent flock, ileum, caecum

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INTRODUCTION

Studies describing gut microbiota in different hosts under various experimental conditions are very common these days due to the simplicity of large scale DNA sequencing. Unfortunately, the real biological meaning of some of these studies might be questionable since many of the experiments are performed once without repetition and random fluctuations in the composition of gut microbiota between experimental and control groups, independent of the studied intervention, cannot be excluded. Moreover, output of these experiments is dependent on the composition of gut microbiota in experimental animals just prior to the intervention. The association of a particular intervention and its positive or negative effect on particular species is therefore difficult to verify and reproduce by other authors especially when it is not known what is microbiota of normal or abnormal composition. In addition, gut microbiota is mostly determined from fecal material due to the non-invasive access to this type of sample, or from the cecal or colonic contents due to the high microbiota complexity of these samples (Videnska et al., 2014a, b; Gao et al., 2017; Xiong et al., 2018). Microbiota in the small intestine of chickens is less commonly characterized (Borda-Molina et al., 2016; Xu et al., 2018) despite the fact that substantial nutrient resorption occurs in the small intestine and that its proper functioning with correct microbiota may considerably affect host performance.

Chickens in commercial production represent a specific case of host-microbiota interactions since these are hatched in a clean hatchery environment in the absence of any contact with adult hens, which would act as a source of optimal microbiota. The development of gut microbiota in commercial chickens is therefore dependent exclusively on environmental sources. These may differ from batch to batch and consequently, gut microbiota may differ among different batches and flocks. This is also in agreement with observations that the cecum of newly hatched chickens can be easily populated by microbiota of a different composition from the very first day of their life (Polansky et al., 2016; Varmuzova et al., 2016; Siegerstetter et al., 2018). Development of microbiota in commercially hatched chickens will therefore slightly differ among chickens originating from different batches and this variation may then affect overall flock performance. However,

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especially in the cases of partially decreased flock performance but without clear clinical signs it is difficult to select appropriate laboratory test for finding a reason of compromised performance. In such cases, unbiased "omics" tests like nextgen sequencing of variable regions of 16S rRNA may represent an interesting option. This is why in this study, we used DNA sequencing to characterize chicken gut microbiota in flocks of optimal and compromised performance. In one of the batches with suboptimal performance, we identified an increased abundance of *Helicobacter* in the ileum and in other samples of adult hens from a flock with decreasing egg production we identified an enrichment of *Fusobacterium* in the cecum. Although none of the identified unusual microbiota members represented a well-recognized chicken pathogen, their overgrowth can be associated with decreased performance and these bacterial species may represent new opportunistic chicken pathogens, the zoonotic potential of which cannot be excluded.

MATERIALS AND METHODS

Experimental Animals and Sample Collection

COBB broiler parents originated from a local farm from four different flocks which were monitored between March 2015 and January 2017. Microbiota development was monitored starting at 5 or 6 wk old (depending on each batch) and ending at 14 to 20 wk old chickens depending on the owner's decision when the flocks were transported to another location for egg production. In each batch, chickens from 2 different buildings within the same farm were taken. Usually 6 chickens (3 chickens from each building) were taken for each time point. However, when collecting samples in the last batch, 12 chickens per time point were collected. Additionally, we sampled 10 adult hens, 53 wk of age, from the same broiler parent line in which egg production dropped. After chickens were sacrificed under chloroform anesthesia, cecal and ileal contents were collected and frozen at −20°C for microbiota characterization. Cecal contents were always collected and ileal contents were collected in the chickens from flocks 2 and 4 as well as from the adult hens. Although the sampling was not performed at exactly the same time points in each batch, we always covered the whole period of rearing. In total, 160 cecal samples and 100 ileal contents were processed in this study (Table 1).

Microbiota Characterization by Sequencing the V3/V4 Region of 16S rRNA Genes

Cecal and ileal content samples were homogenized in a MagNALyzer (Roche) with the use of zirconia/silica beads (BioSpec Products). Following homogenization, the DNA was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen). The DNA concentration was determined spectrophotometrically (NanoDrop 2000c, Thermo Scientific) and DNA was stored at −20°C until use. DNA samples were used as a template in PCR with forward primer 5′- *TCGTCGGCAGCGT CAGATGTGTATAA GAGACAG*-MID-GT-CCTACGG GNGGCW GCAG-3′ and reverse primer 5′- *GTCTCGTGGGCT CGGAGATGTGT ATAAGAGACAG*-MID-GT-GACTACHVGGGTATCTAATCC-3′. The sequences in italics served as index and adapter ligation sequences whereas the underlined sequences allowed for amplification over the V3/V4 region of 16S rRNA genes. MIDs represent different sequences of 5, 6, 7, or 9 base pairs in length which were used to differentiate samples originating from individual chickens. PCR amplification and clean up were performed using KAPA Taq HotStart PCR Kit (Kapa Biosystems) following the protocol for 16S metagenomic sequencing library preparation recommended by Illumina. In the next step, the DNA concentration was determined fluorometrically with Quant-iT^{TM} dsDNA Assay Kit High Sensitivity (Invitrogen Life Technologies) using Synergy Hybrid reader H1 (Biotek). The DNA was diluted to 100 ng/μL, groups of 12 to 14 PCR products with different MID sequences were pooled and indexed with a Nextera XT Index Kit (Illumina) using KAPA HiFi HotStart ReadyMix (Kapa Biosystems). Prior to sequencing, the concentration of differently indexed samples was determined using the KAPA Library Quantification Complete kit (Kapa Biosystems). All indexed samples were diluted to 4 nM, denatured with 0.2 M NaOH and phiX DNA was added to a final concentration of 20%. Sequencing was performed using MiSeq® Reagent Kit and MiSeq System according to the manufacturer's instructions (Illumina).

Sequence Processing and Classification of Obtained Sequences into Appropriate Taxa

The fastq files generated as an Illumina sequencing output were uploaded into Qiime software (Caporaso et al., 2010) and reverse reads from paired-end sequencing were joined. Quality trimming criteria were set to a value of 19 and no mismatch in the MID sequences. In the next step, chimeric sequences were predicted by the slayer algorithm (Chimeraslayer) and excluded from further analysis. The resulting sequences were then classified with RDP Seqmatch with an operational taxonomic unit (**OTU**) discrimination level set to 97%. Principal coordinate analysis implemented in Qiime was used for data visualization. Diversity indices, numbers of sequences per sample and taxonomic classification into families and genera were obtained directly from the Qiime software.

Table 1. List of samples collected and processed in this study.

Age (weeks)	3/2015–6/2015 Flock 1 broiler parent Caecum	8/2015–11/2015 Flock 2 broiler parent Caecum	Ileum	1/2016–4/2016 Flock 3 broiler parent Caecum	11/2016–1/2017 Flock 4 broiler parent Caecum	Ileum	5/2017 Flock 5 laying hens Caecum	Ileum
5	6	6	6		12	12		
6				6				
7		6	6					
8					12	12		
9		6	6(1)	6				
10	6							
11		6	6(1)		12	12		
12				6				
13		6	6					
14	6				12	12		
15		6	6	6				
17	6	6	6					
18								
20	6			6				
53							10	10
Total	30	42	42	30	48	48	10	10

(1) These 2 individual samples were excluded from the analysis due to technical reasons.

RESULTS

Flock Description

This study was performed with the aim to compare the microbiota composition in chickens from different flocks that were raised at the same farm in four consecutive batches, starting with sample collection from the first flock in March 2015 and ending with the last (fourth) flock in January 2017. Since the four batches were raised at the same farm of the same owner, general management was the same for each of them. We expected that each flock would have a slightly different performance. Indeed, flock 4 was of poorer performance and the owner decided to move it to a different location as early as at week 15 of life. All the remaining flocks were monitored until week 18 to 20 when the chickens were moved to another location for egg laying. In addition, we also sampled 53-wk-old hens from a broiler parent flock in which egg production dropped considerably. Samples from flock 4 and from 53-wk-old hens could be therefore considered as originating from birds with suboptimal performance.

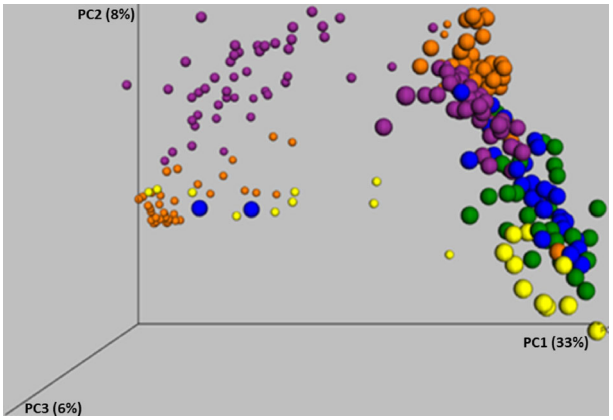

Figure 1. Composition of gut microbiota in all chickens visualized by weighted PCoA. Microbiota in the ileum differed from microbiota in the cecum since ileal samples (small symbols) separated from cecal samples (large symbols). Except for cecal microbiota in flocks 1 and 3 (blue and green color, respectively), the remaining flocks formed separated clusters, i.e., chickens belonging to the same flock shared similar microbiota but different from other flocks. Samples from two chickens in flock 1 must have been taken shortly after filling the cecum with fresh contents from the ileum since these 2 samples clustered outside the cecal samples but close to the ileal samples (2 big blue spots among the other small spots). Blue color—samples from flock 1, orange—flock 2, green—flock 3, magenta—flock 4, yellow – 53-wk-old hens.

Sequencing Coverage and Depth

Out of 260 samples, we failed to analyze successfully two ileal samples (Table 1). For the remaining 258 samples, we obtained 5,142,235 reads, which represents an average coverage of 19,931 reads per sample. The lowest coverage of a sample in this study was 296 reads and the sample with the highest coverage was characterized by 161,614 reads. Ten samples were characterized by less than 1,000 reads and 24 samples were characterized by less than 2,000 reads. Out of these 24 samples, 22 originated from ileal samples, i.e., we experienced greater difficulties with obtaining reasonable data from the ileum than from the cecum. The lower coverage in these samples did not affect the conclusions of this study due to the lower complexity of ileal microbiota.

General Microbiota Characterization

Principal coordinate analysis of all samples separated those of ileal and cecal origin (Figure 1). In addition, except for cecal microbiota in flocks 1 and 3, chickens from the same flock were colonized by microbiota of similar composition but different from other flocks since

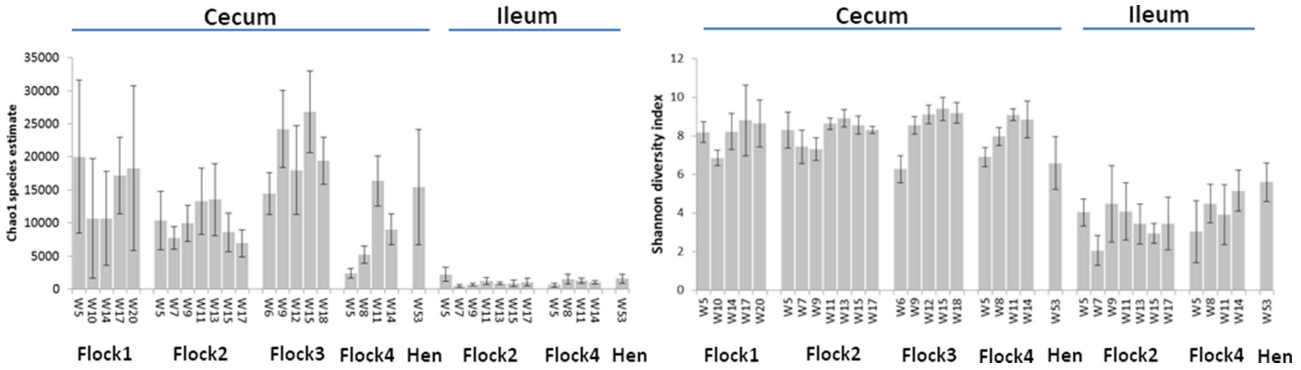

Figure 2. Diversity of microbiota in the ileum and cecum. Chao1 estimate of species forming ileal microbiota was nearly 10 times lower than the number of species colonizing the cecum. Shannon indices confirmed the lower diversity of ileal microbiota compared to cecal microbiota.

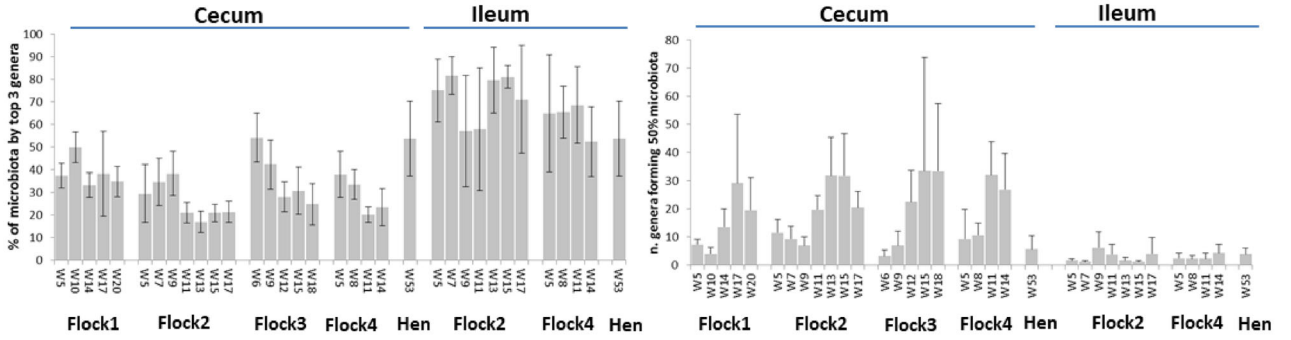

Figure 3. Microbiota in the cecum is more diverse than in the ileum but requires more time to reach its final composition. Top 3 genera formed over 60% of all ileal microbiota but around 30% of cecal microbiota (left panel). Ileal microbiota is therefore dominated by a limited number of key colonizers. This correlates with N_m50 values (right panel). Less than 5 genera formed more than 50% of all microbiota in ileal samples and this did not change over time. On the other hand, higher N_m50 values were recorded for cecal samples and these increased with age. The low N_m50 value for cecal microbiota of 53-week-old hens was influenced by a high abundance of Fusobacterium in these samples.

these formed separated clusters, which only slightly overlapped with the others (Figure 1).

Microbiota Alpha Diversity in the Ileum and Cecum

Differences in the composition of ileal and cecal microbiota were further confirmed following comparison of diversity indices. The number of estimated species in the cecum fluctuated around 10,000. On the other hand, a Chao1 estimate of alpha diversity in the ileum was around 1,100, i.e., approx. 10 times lower (Figure 2). Shannon indices for chicken cecal and ileal samples from different flocks and of different age also confirmed the lower alpha diversity in microbiota inhabiting the ileum compared to the cecum (Figure 2). For all samples, we also checked the proportion of the 3 most abundant genera. In the ileum, the 3 most abundant genera formed on average 62.5% of all microbiota. On the other hand, the top 3 genera formed on average only 29.8% of all microbiota in the cecum (Figure 3). Alternatively, we determined how many genera were needed to define 50% of the total cecal or ileal microbiota (N_m50). In ileal microbiota, 1 to 5 genera were enough to form 50% of all microbiota while the N_m50 values for cecal samples ranged from 3 to 32. In addition, the N_m50 index remained constant in ileal samples but increased with age in the cecal samples indicating increasing diversity in the cecal microbiota composition.

Identification of Bacterial Families Characteristic for Microbiota from the Chicken Ileum and Cecum

Gram-positive bacteria belonging to phylum *Firmicutes* populated the ileum of well-performing chickens. These included mainly families *Peptostreptococcaceae* in the flocks of chickens before reaching sexual maturity around week 18 of life and *Lactobacillaceae* in adult hens, although families *Erysipelotrichaceae* and *Clostridiaceae* 1 were also common to the ileum. The ileum of chickens in less-well performing flock 4 was characterized by the presence of *Helicobacteraceae* (Figure 4). A single OTU was responsible for the high abundance of *Helicobacteraceae* and manual BLAST comparison of the sequence of this OTU with 16S rRNA GenBank database identified this OTU as *Helicobacter brantae*.

Cecal microbiota was dominated by *Bacteroidetes* and *Firmicutes*. Dominant families within *Firmicutes* were different from those in the ileum and included

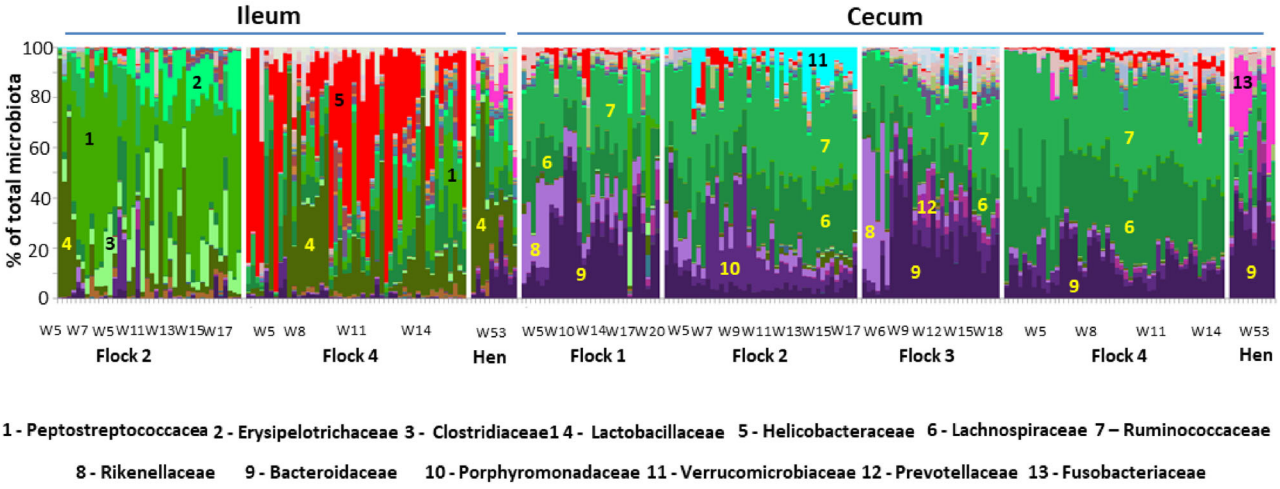

Figure 4. Bacterial families forming ileal and cecal microbiota of chickens and hens. Microbiota of all individual chickens is shown in this figure. Chicken age in monitored flocks is shown in weeks (w5 to w53) and only major families are highlighted. Families belonging to Firmicutes are highlighted with shades of green and families of phylum Bacteroidetes are highlighted in shades of purple.

Lachnospiraceae and *Ruminococcaceae*. Phylum *Bacteroidetes* was represented by families *Rikenellaceae*, *Bacteroidaceae*, *Porphyromonadaceae*, and *Prevotellaceae*. *Rikenellaceae* were detected in high abundance mainly in chickens around 5–6 weeks of age (Figure 4). The cecal microbiota of flock 2 was characterized by a high abundance of *Verrucomicrobiaceae* and the cecal microbiota of poorly performing adult hens was characterized by a high abundance of *Fusobacteriaceae* (*Fusobacterium mortiferum* after manual BLAST comparison with 16S rRNA GenBank database). This bacterium formed 19.7% of total microbiota on average but in 3 out of 10 tested hens, its abundance exceeded 30% of all cecal microbiota (Figure 4).

DISCUSSION

In this study, we monitored ileal and cecal microbiota in four consecutive batches of broiler parent flocks. Such a study is always more complicated to perform since the samples are collected over a long period and it is impossible to predict whether specific patterns of microbiota development will be recorded at all. This is also a reason why samples were not collected in exactly the same time points.

Although we collected ileal samples in only two batches, the general comparison showed extensive differences between microbiota in the ileum and cecum, similar to previous studies (Borda-Molina et al., 2016; Munyaka et al., 2016; Ranjitkar et al., 2016; Siegerstetter et al., 2017). Ileal microbiota was dominated by Gram-positive bacteria while Gram-negative bacteria formed a substantial part of the gut microbiota only in the cecum. However, there were differences also in Gram-positive microbiota from the ileum and cecum. Ileal microbiota was formed by *Peptostreptococcaceae*, *Lactobacillaceae*, *Erysipelotrichaceae*, and *Clostridiaceae* 1, while Gram-positive bacteria in the cecal microbiota belonged mainly to families *Lachnospiraceae* and *Ruminococcaceae*. We also noticed that the ileal microbiota of chickens prior to sexual maturity was dominated by *Peptostreptococcaceae*, while the ileal microbiota of adult hens was dominated by *Lactobacillaceae*. It is possible that this change is a consequence of hormonal changes at the onset of egg laying, induction of specific proteins like vitelogenin (Volf et al., 2016) or increased demand for calcium resorption. This shift in major colonizers of the small intestine could be also caused by changes in nutrition at the onset of egg laying. Interestingly, except for a switch in the dominance of two main genera, microbiota in the small intestine remained rather constant and did not develop with chicken age. This was different from the microbiota in the cecum where microbiota developed with increasing age (Lu et al., 2003; Videnska et al., 2014b; Awad et al., 2016). In 2 batches, we recorded that *Rikenellaceae* was the first family of phylum *Bacteroidetes* which appeared in the cecum and which was later replaced with representatives of families *Bacteroideaceae*, *Porphyromonadaceae*, and *Prevotellaceae*. This seems to be a common developmental profile since high *Rikenellaceae* abundance was recorded in several studies in 3- to 5-wk-old chickens (Torok et al., 2011; Li et al., 2016; Costa et al., 2017).

None of the monitored batches of flocks exhibited clinical signs of any infection. However, despite the absence of clinical signs, flock 4 and the adult hens were of suboptimal performance. The chickens in flock 4 exhibited a low body weight increase and the owner decided to move this flock to another location as early as 15 wk old, i.e., a month earlier than the remaining 3 flocks. The cecal microbiota in this flock did not exhibit any extensive differences from chickens in other flocks but ileal microbiota was dominated by *Helicobacter*. Little is known about *Helicobacter* infections in chickens. Moreover, classification of genus *Helicobacter* is still developing and our identification of

H. brantae as the species of increased abundance in the ileum of chickens in flock 4 must be therefore taken with a certain care. Furthermore, the interactions of a particular host and *Helicobacter* are dependent not only on species but even particular clones within species (Flahou et al., 2012). Despite this, *H. brantae* was detected in wild birds at a relatively low incidence (Fox et al., 2006; Garcia-Amado et al., 2013). The former study also considered the zoonotic potential of *H. brantae* (Fox et al., 2006). There is a report on infection of chickens with *Helicobacter* with an asymptomatic course of infection but with mild lesions in the caeca of sacrificed chickens (Ceelen et al., 2007). Another report showed the presence of *Helicobacter pullorum* in chicken cecal microbiota using metagenomic sequencing (Sergeant et al., 2014).

Microbiota of adult hens differed from that of chickens before reaching sexual maturity. *Lactobacillus* dominated over *Peptostreptococcus* in the ileum, similar to previous reports (Borda-Molina et al., 2016; Xu et al., 2018). However, the dominance (around 20% of cecal microbiota) of *Fusobacterium* in the cecum of hens was quite unexpected, as *Fusobacterium* is not commonly found among chicken gut microbiota members. We cannot conclude whether this was the cause of decreased egg production or a consequence of some other factor, which resulted *Fusobacterium* overgrowth. *F. mortiferum* has been previously associated with multifactorial diseases. *Fusobacterium mortiferum* was isolated from a human patient with multibacterial sepsis (Matsukawa et al., 2003) and from mixed anaerobic infection of the thyroid gland (Stavreas et al., 2005). Though mostly appearing as a commensal, if present in immunocompromised individuals, *F. mortiferum* may spread beyond the intestinal tract and induce sepsis and an inflammatory response in its host.

In this study, we monitored the development of ileal and ceacal microbiota in broiler parent flocks from week 5 of life until sexual maturity, and in adult hens of the same genetic line in which egg production dropped below an economical profit ratio. We found that there were rather wide borders for the composition of gut microbiota as each flock developed slightly different microbiota. Though we identified two bacterial species as potentially novel opportunistic pathogens of chickens, their real significance for poultry gut health will have to be determined by experimental infections. Despite this, we have shown that next generation sequencing can be used in the cases with non-specific symptoms like suboptimal body weight increases or decrease in egg production. In such cases, it might be difficult to decide for appropriate laboratory test while an unbiased sequencing may more likely point towards the most likely causative agent.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

REFERENCES

Awad, W. A., E. Mann, M. Dzieciol, C. Hess, S. Schmitz-Esser, M. Wagner, and M. Hess. 2016. Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with *Campylobacter jejuni* infection. Front. Cell. Infect. Microbiol. 6:154.

Borda-Molina, D., M. Vital, V. Sommerfeld, M. Rodehutscord, and A. Camarinha-Silva. 2016. Insights into Broilers' gut microbiota fed with phosphorus, calcium, and phytase supplemented diets. Front. Microbiol. 7:2033.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, 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.

Ceelen, L. M., A. Decostere, K. Chiers, R. Ducatelle, D. Maes, and F. Haesebrouck. 2007. Pathogenesis of *Helicobacter pullorum* infections in broilers. Int. J. Food Microbiol. 116:207–213.

Costa, M. C., J. A. Bessegatto, A. A. Alfieri, J. S. Weese, J. A. Filho, and A. Oba. 2017. Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PLoS One. 12:e0171642.

Flahou, B., K. V. Deun, F. Pasmans, A. Smet, J. Volf, I. Rychlik, R. Ducatelle, and F. Haesebrouck. 2012. The local immune response of mice after *Helicobacter suis* infection: strain differences and distinction with *Helicobacter pylori*. Vet. Res. 43:75.

Fox, J. G., N. S. Taylor, S. Howe, M. Tidd, S. Xu, B. J. Paster, and F. E. Dewhirst. 2006. *Helicobacter anseris* sp. nov. and *Helicobacter brantae* sp. nov., isolated from feces of resident Canada geese in the greater Boston area. Appl. Environ. Microbiol. 72:4633–4637.

Gao, P., C. Ma, Z. Sun, L. Wang, S. Huang, X. Su, J. Xu, and H. Zhang. 2017. Feed-additive probiotics accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. Microbiome. 5:91.

Garcia-Amado, M. A., V. Sanz, L. M. Martinez, M. Contreras, M. Lentino, and F. Michelangeli. 2013. Low occurrence of Helicobacter DNA in tropical wild birds, Venezuela, J. Wildl. Dis. 49:991–995.

Li, Y., Q. Xu, Z. Huang, L. Lv, X. Liu, C. Yin, H. Yan, and J. Yuan. 2016. Effect of *Bacillus subtilis* CGMCC 1.1086 on the growth performance and intestinal microbiota of broilers. J. Appl. Microbiol. 120:195–204.

Lu, J., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee. 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. 69:6816–6824.

Matsukawa, Y., N. Kitamura, M. Kaneko, D. Yoshioka, T. Miki, S. Nishinarita, T. Horie, N. Hosokawa, Y. Iwasaki, K. Kumasaka, and K. Kawano. 2003. Multibacterial sepsis in an alcohol abuser with hepatic cirrhosis. Intern. Med. 42:208–210.

Munyaka, P. M., N. K. Nandha, E. Kiarie, C. M. Nyachoti, and E. Khafipour. 2016. Impact of combined ?-glucanase and xylanase enzymes on growth performance, nutrients utilization and gut microbiota in broiler chickens fed corn or wheat-based diets. Poult. Sci. 95:528–540.

Polansky, O., Z. Sekelova, M. Faldynova, A. Sebkova, F. Sisak, and I. Rychlik. 2016. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Appl. Environ. Microbiol. 82:1569–1576.

Ranjitkar, S., B. Lawley, G. Tannock, and R. M. Engberg. 2016. Bacterial succession in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 82:2399–2410.

Sergeant, M. J., C. Constantinidou, T. A. Cogan, M. R. Bedford, C. W. Penn, and M. J. Pallen. 2014. Extensive microbial and functional diversity within the chicken cecal microbiome. PLoS One. 9:e91941.

Siegerstetter, S. C., R. M. Petri, E. Magowan, P. G. Lawlor, Q. Zebeli, N. E. O'Connell, and B. U. Metzler-Zebeli. 2018. Fecal microbiota transplant from highly feed-efficient donors shows little effect on age-related changes in feed-efficiency-associated fecal microbiota from chickens. Appl. Environ. Microbiol. 84:e02330–17.

Siegerstetter, S. C., S. Schmitz-Esser, E. Magowan, S. U. Wetzels, Q. Zebeli, P. G. Lawlor, N. E. O'Connell, and B. U. Metzler-Zebeli. 2017. Intestinal microbiota profiles associated with low and high residual feed intake in chickens across two geographical locations. PLoS One. 12:e0187766.

Stavreas, N. P., C. D. Amanatidou, E. G. Hatzimanolis, I. Legakis, G. Naoum, E. Lakka-Papadodima, G. Georgoulias, P. Morfou, and S. Tsiodras. 2005. Thyroid abscess due to a mixed anaerobic infection with *Fusobacterium mortiferum*. J. Clin. Microbiol. 43:6202–6204.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. Appl. Environ. Microbiol. 77:5868–5878.

Varmuzova, K., T. Kubasova, L. Davidova-Gerzova, F. Sisak, H. Havlickova, A. Sebkova, M. Faldynova, and I. Rychlik. 2016. Composition of gut microbiota influences resistance of newly hatched chickens to *Salmonella* Enteritidis infection. Front. Microbiol. 7:957.

Videnska, P., M. M. Rahman, M. Faldynova, V. Babak, M. E. Matulova, E. Prukner-Radovcic, I. Krizek, S. Smole-Mozina, J. Kovac, A. Szmolka, B. Nagy, K. Sedlar, D. Cejkova, and I. Rychlik. 2014a. Characterization of egg laying hen and broiler fecal microbiota in poultry farms in Croatia, Czech Republic, Hungary and Slovenia. PLoS One. 9:e110076.

Videnska, P., K. Sedlar, M. Lukac, M. Faldynova, L. Gerzova, D. Cejkova, F. Sisak, and I. Rychlik. 2014b. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. PLoS One. 9:e115142.

Volf, J., O. Polansky, K. Varmuzova, L. Gerzova, Z. Sekelova, M. Faldynova, V. Babak, M. Medvecky, A. L. Smith, B. Kaspers, P. Velge, and I. Rychlik. 2016. Transient and prolonged response of chicken cecum mucosa to colonization with different gut microbiota. PLoS One. 11:e0163932.

Xiong, W., Y. Wang, Y. Sun, L. Ma, Q. Zeng, X. Jiang, A. Li, Z. Zeng, and T. Zhang. 2018. Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. Microbiome. 6:34.

Xu, S., Y. Lin, D. Zeng, M. Zhou, Y. Zeng, H. Wang, Y. Zhou, H. Zhu, K. Pan, B. Jing, and X. Ni. 2018. *Bacillus licheniformis* normalize the ileum microbiota of chickens infected with necrotic enteritis. Sci. Rep. 8:1744.