RESEARCH ARTICLE

Overfeeding and genetics affect the composition of intestinal microbiota in *Anas platyrhynchos* (Pekin) and *Cairina moschata* (Muscovy) ducks

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MICROBIOLOGY ECOLOGY

FEMS

Abstract

To investigate the effect of overfeeding on the ileal and cecal microbiota of two genotypes of ducks (Pekin and Muscovy), high-throughput 16S rRNA gene-based pyrosequencing was used. The ducks were overfed for 12 days with 58% maize flour and 42% maize grain. Samples were collected before the overfeeding period (at 12 weeks), at 13 weeks, at 14 weeks, and 3 h after feeding. In parallel, ducks fed *ad libitum* were killed at the same ages. Whatever the digestive segment, the genotype, and the level of intake, *Firmicutes* and *Bacteroidetes* are the dominant phyla in the bacterial community of ducks (at least 80%). Before overfeeding, ileal samples were dominated by *Bacilli*, *Clostridia*, and *Bacteroidia* classes (≥ 70%), and cecal samples, by *Bacteroidia* and *Clostridia* classes (around 90%) in both Pekin and Muscovy ducks. The richness and diversity decreased in the ileum and increased in the ceca after overfeeding. Overfeeding triggers major changes in the ileum, whereas the ceca are less affected. Overfeeding increased the relative abundance of *Clostridiaceae*, *Lactobacillaceae*, *Streptococcaceae*, and *Enterococcaceae* families in the ileum, whereas genotype affects particularly three families: *Lachnospiraceae*, *Bacteroidaceae*, and *Desulfovibrionaceae* in the ceca.

Introduction

The intestinal microbiota plays a major role in animal's health and host physiology (Backhed *et al.*, 2004), for example, in immunological development or nutrient utilization (Gordon & Pesti, 1971). Microbiota interacts with the intestinal mucosa and can modify their structures and activity (Taschuk & Griebel, 2012). Different stresses experienced by animals such as a change in diet can modify the composition of the microbiota (Serino *et al.*, 2011) as happens when overfeeding ducks during the fatty liver ('foie gras') production. During this period, the animals are overfed exclusively with maize, which is rich in carbohydrates (especially starch), to induce a hepatic steatosis resulting in the storage of fatty acids in the liver. This unbalanced diet could affect the equilibrium of the

gut bacterial community. Indeed, the digestive tract is colonized by a large number of bacterial species. In chickens, the bacterial activity is more intense in the crop, the ceca, and small intestine in comparison with proventriculus gizzard and pancreas (Fuller, 1984). Most of the bacteria identified by clone library analysis belong to the phyla *Bacteroidetes* and *Firmicutes* (Zhu *et al.*, 2002; Lu *et al.*, 2003; Ahmed *et al.*, 2008). Studies in classical microbiology and molecular biology show that the three segments of the small intestine contain facultative anaerobic bacteria, while the ceca contain strict anaerobic bacteria (Mead & Adams, 1975; Zhu *et al.*, 2002; Lu *et al.*, 2003). Lactobacilli are the major bacterial population in the small intestine (duodenum, jejunum, and ileum), whereas *Clostridium* spp. and *Bacteroides* spp. (obligate anaerobes) are dominant in the ceca with both

culture-dependent and molecular methods (Zhu *et al.*, 2002; Lu *et al.*, 2003).The microbiota of several bird species has been studied by molecular analysis, but not in ducks (Kohl, 2012). The objective of this study was to identify the microbiota composition of two genotypes of ducks, Pekin (*Anas platyrhynchos*), which are used for their rapid growth and reproduction, and Muscovy (*Cairina moschata*), which have a high capacity for fat storage. It has been shown that during overfeeding, Pekin ducks tend to achieve significant storage in peripheral tissues, while the liver storage is less than in others species (Davail *et al.*, 2003a; Hermier *et al.*, 2003) and suggested that lipoprotein lipase (LPL), which hydrolyzes the lipoproteins rich in triacylglycerol, can partly explain lipid distribution between liver and hepatic tissues (Davail *et al.*, 2003b). Interestingly, the LPL activity remains high during the overfeeding period in Pekin ducks and dramatically decreases in Muscovy ducks. This study is the first inventory of intestinal microbiota in ducks in both ileum and ceca. Furthermore, the genetic impact was evaluated using the two genotypes Muscovy and Pekin, and the effect of overfeeding to know the impact of a food stress on the microbiota was studied using a FLX amplicon pyrosequencing.

Material and methods

Experimental design

All experimental procedures involving ducks were in accordance with the French national guidelines for the care of animal for research purposes. Male ducks were raised in a breeding structure belonging to the French National Research Institute for Agronomy (INRA) at the 'domaine d'Artiguères', Benquet, France. From the first day of life to 4 weeks of age, they were fed *ad libitum* with 2-mm granules on a diet providing 11.93 MJ kg^{-1} of food and crude protein (CP) 17.5% ('starter diet'). From 5 to 12 weeks of age, the birds were fed with 4-mm granules on a restricted diet providing 11.72 MJ kg^{-1} of food and CP 15.5%. The composition of the different feeds used in this study is listed in Table 1. At 12 weeks of age, all animals were weighed, and 25 ducks were selected. Of these, three of each genotype were killed before overfeeding. The remaining 12 animals (called overfed ducks) were fed in cages containing four ducks. They were hydraulically fed with food consisting of 58% maize flour and 42% maize grain ('overfeeding feed'). The digestive contents from three ducks of each treatment and each genotype were removed at 13 weeks (meal 12) and at 14 weeks (meal 24), 3 h after feeding. Furthermore, at 12 weeks, seven ducks (called not-overfed) were fed normally with 4-mm granules on a restricted diet

providing 11.72 MJ kg^{-1} of food and CP 15.5%. Finally, the animals were killed at 13 and 14 weeks (the same age as overfed ducks).

Sampling for microbiota analysis

The ducks were killed by exsanguination after electric stunning, 3 h following the last meal to homogenize the filling level of the ducks' digestive tract. Ducks of each genotype with similar weight were selected at three times: at 12 weeks (before overfeeding), 13 weeks (meal 12), and 14 weeks (meal 24). Ileum and ceca were immediately collected and kept on ice. The digestive contents of the ileum and ceca were collected by gently squeezing the organ. The digestive contents of each animal were studied individually and were stored at −20 °C for short-time molecular analysis.

DNA extraction and high-throughput sequencing and analysis of 16S rRNA gene amplicons

Total DNA from ileal and cecal samples was extracted using the QIAamp DNA stool minikit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer with 220 mg as starting material. An additional lysis step is made using lysozyme (Sigma, Saint-Louis, MO) to improve the DNA extraction of the gram-positive bacteria present within the samples (Johansen *et al.*, 2007). The extracted DNA (from culture or digestive contents) was eluted in 200 μL of elution buffer and stored at −20 °C until real-time PCR analysis. Amplicons from the V3 to V4 regions of 16S rRNA genes (460 bp on *Escherichia coli*, GenBank accession number J01695) of day 12 samples were amplified using bacterial forward 343F (TACGGRAGGCAGCAG; Liu *et al.*, 2007) and reverse 784R (TACCAGGGTATCTAATCCT; Andersson *et al.*, 2008) primers. Each primer had a barcode sequence of ten nucleotides at the 5′ end, which was unique for each sample. The preparation of amplicons was performed in a total volume of 100 μL containing 1× PCR buffer, 200 μM of dNTP, 1 U Iysis DNA polymerase (MP Biomedicals), 0.5 μM of each primers, and 1–5 ng of DNA template. The amplification program consisted of an initial denaturation step at 94 °C for 2 min; 32 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s; and a final extension step at 72 °C for 7 min. The PCR products were purified with the QIAquick PCR Purification kit (Qiagen) followed by DNA yield quantification and quality estimation using a NanoDrop ND-100 spectrophotometer. The size of the PCR products was confirmed by gel electrophoresis, and then, the purified PCR products were quantified using the Quant-iT

Table 1. Ingredients and chemical composition of the experimental diets

Starter diet

Ingredients		Nutrient		Overfeeding feed (dry corn) DM%*	
Wheat	35	ME Vol kg^{-1}	2085	ME Vol kg^{-1}	–
Corn	38.69	Humidity	12.61	Humidity	–
Rapeseed oil free	12	CP	15	CP	8.91
Sunflower cake	11.35	Fat	2.26	Fat	3.68
Sodium carbonate	1.375	Cellulose	5.28	Cellulose	2.77
Dicalcium phosphate	0.575	Ashes	4.99	Ashes	–
Lysine	0.298	Starch	47.32	Starch	75.91
Salt	0.36	*Nutrient level (% of raw material)*		*Nutrient level (% of raw material)*	
Methionine	0.086	Lysine	0.701	Lysine	–
Choline chloride	0.04	Methionine	0.36	Methionine	–
Premix + vitamin	0.23	Meth + cystine	0.705	Meth + cystine	–
		Threonine	0.548	Threonine	–
		Tryptophane	0.173	Tryptophane	–
		Linoléic Ac	1.2	Linoléic Ac	–
		Calcium %	0.95	Calcium	–
		Phosphorus	0.519	Phosphorus	–
		Sodium	0.15	Sodium	–
		Available Phosphorus	0.299	P.Disp Vo	–
		pH	–	pH	5.36

CP, crude protein.
*Dry matter %.

PicoGreen dsDNA Assay Kit (Invitrogen, Saint Aubin, France) on a ABI prism 7900 HT sequence detection system (Life technology, Invitrogen, A-BIOSYSTEM, Villebon-sur-Yvette, France). The sequencing of the 16S rRNA genes was performed by 454 GS FLX (454 Life Sciences – Roche) and Titanium chemistry according to the manufacturer's instructions. Amplicons of 450 bp were pooled using equal amounts of each PCR product. For the analysis, samples were recognized by couples of tag sequence, which had been fixed on the universal primers provided by Roche during the amplification.

Pyrotag handling and analysis

A total of 498 772 16S rRNA gene sequences (also referred to as 16S pyrotags) were obtained from 454 Titanium pyrosequencing run for the 49 samples. The 16S pyrotags were sorted on the basis of their respective barcodes to form a total of 49 pyrotag library representing the 49 collected ileal and cecal samples. Sequences were sequentially filtered using a Python script developed by the bioinformatic platform of Toulouse, first removing those sequences with a short sequencing length (< 150 nt; 56 393 sequences removed), those with at least one ambiguous base (13 759 sequences removed) or with a long homopolymer (> 8; 206 sequences), those which did not match the proximal PCR primer sequences (with two mismatches allowed; 1441 sequences removed), and finally those having both primers, but with a length

shorter than 350 pb (912 sequences removed). A total of 426 061 sequences were retained corresponding to 9582 ± 5624 sequences per samples.

Taxonomical classification and statistical analysis

Filtered sequences were analyzed using MOTHUR software, version 1.24 (Schloss *et al.*, 2009). Readings were aligned over the SILVA alignment database provided by MOTHUR software (14 956 sequences corresponding to the unique sequences in the SSU REF database v102; Pruesse *et al.*, 2007), and an alignment quality was calculated using the SILVA secondary structure map file (1072 sequences were removed). After calculating a pairwise distance between aligned sequences, they were clustered into operational taxonomic units (OTU, cutoff of 0.05 using a furthest neighbor clustering). Rarefaction curves, abundance-based coverage estimator (ACE), and Chao1 richness were calculated including rarefaction and Chao1 estimator. The Shannon diversity index was calculated according to Hayek & Buzas (1996). The sequences were analyzed using R (http://www.r-project.org/) to obtain the results of the composition of the microbiota in our treatment conditions. Statistical analysis of similarity (ANOSIM) between band patterns was carried out using 10 000 permutations. The ANOSIM R-value indicated the extent to which the groups differed ($R > 0.75$, well-separated groups; $0.50 < R < 0.75$, separated but overlapping

groups; and 0.25 < R < 0.50, separated but strongly overlapping groups). These proximity values were also graphically explored by nonmetric multidimensional scaling (nMDS). When values are given in the text, they are expressed as mean ± SEM.

Results

Ileal microbial community in Pekin and Muscovy ducks

The microbial diversity in ileal samples of Pekin and Muscovy ducks was estimated by calculating the number of OTUs. In Pekin ducks, the number of OTUs with a cutoff of 0.05 was 1147 ± 638 with coverage per sample of 91.1 ± 3.3%. The average number of sequences was 5202 ± 3435, and 12 different phyla were listed for 184 different taxa. Furthermore, the Chao1, the ACE, and the Shannon index were 2167 ± 509, 3114 ± 747, and 4.0 ± 0.5, respectively (Table 2). In Muscovy ducks, 1022 ± 275 OTUs with a cutoff of 0.05 were detected. The average number of sequences per sample was 11 533 ± 1944, and the sequences were affiliated with nine phyla and 214 different taxa. The different diversity indices for these samples before overfeeding were 1816 ± 384 for the Chao1 indices, 2420 ± 510 for ACE, and 5.2 ± 0.8 for the Shannon diversity index (Table 2). The two major phyla in both Pekin and Muscovy ducks were *Firmicutes* (71.5 ± 13.2% and 48.7 ± 11.9%, respectively) and *Bacteroidetes* (16.4 ± 15.3% and 21.8 ± 12.3%, respectively). *Proteobacteria* represented around 10.6 ± 6.0% in Pekin and 26.4 ± 9.6% in Muscovy (Fig. 1a). Finally, other phyla such as *Actinobacteria*, *Fusobacteria*, *Deferribacteres*, *Spirochetes*, or *Acidobacteria* represented < 2% or 3.5% of the population in Pekin and Muscovy ducks, respectively (Fig. 1a). To evaluate the microbiota composition at finer taxonomic

levels, class distributions were analyzed. In Pekin ducks, *Firmicutes* were dominated by *Bacilli* (53.6%) and *Clostridia* (22.9%), *Bacteroidia* (from the phylum *Bacteroidetes*) accounted for 16.1%, and the classes *Gammaproteobacteria* and *Deltaproteobacteria* that are part of the *Proteobacteria* were represented at levels of 3.9% and 0.4%, respectively (Fig. 1b). The *Actinobacteria* represented < 1% of the population of the ileum (Fig. 1b). In Muscovy ducks, *Firmicutes* were mainly composed of *Clostridia* (36.7%), *Bacilli* (11.7%), and *Bacteroidia* (21%). Regarding the phylum *Proteobacteria*, the major classes were *Gammaproteobacteria* (18.1%) and *Deltaproteobacteria* (5.6%) (Fig. 1b). Finally, 173 genera were detected in Pekin ducks, and 196 genera, in Muscovy ducks.

Cecal microbial community in Pekin and Muscovy ducks

The average number of ceca OTUs with a cutoff of 0.05 was 901 ± 137 with coverage per sample of 91.1 ± 3.3% in Pekin ducks, whereas there were 1251 ± 569 OTUs in Muscovy ducks (Table 2). The average number of sequences in Pekin was 8626 ± 2614 with eight phyla represented by 111 different taxa and in Muscovy 8784 ± 5979 represented by 118 taxa grouped in eight different phyla. Regarding the Chao1, the ACE, and the Shannon index, the values were 1782 ± 390, 2631 ± 493, and 5.2 ± 0.2, respectively, in Pekin ducks and 2512 ± 469, 3765 ± 706, and 5.8 ± 0.2, respectively, in Muscovy ducks. In Pekin ducks, the cecal microbiota was mainly composed of *Bacteroidetes* (64.8 ± 2.5%) and *Firmicutes* representing 27.5 ± 3.3% of the population, while there was only 3.92 ± 1.2% of *Proteobacteria*. In Muscovy ducks, *Bacteroidetes* (50.9 ± 2.1%), *Firmicutes* (40.5 ± 2.9%), and *Proteobacteria* (7.1 ± 1.3%) were also dominant. Other phyla, such as *Actinobacteria*, *Deferribacteres*, *Spirochetes*, and *Synergistetes*, represented < 2% of

Table 2. Estimators of diversity during overfeeding in ileum and ceca of Muscovy and Pekin ducks

Segment	Ileum				Ceca			
Period	Bof	Mof	Eof	SEM*	Bof	Mof	Eof	SEM*
Muscovy								
Number of operational taxonomic unit	1022	345	444	211	1251	1972	1430	216
Chao1 estimated richness	1816	576	778	384	2512	4014	2722	469
ACE	2420	807	989	510	3765	6032	4096	706
Shannon diversity index	5.2	2.7	2.6	0.8	5.8	5.6	5.2	0.2
Pekin								
Number of operational taxonomic unit	1147	480	279	262	901	1216	1552	187
Chao1 estimated richness	2167	951	453	509	1782	2407	2959	340
ACE	3114	1313	601	747	2631	3651	4326	451
Shannon diversity index	4.0	2.9	2.3	0.5	5.2	5.2	5.5	0.1

ACE, abundance-based coverage estimator; Bof, before overfeeding (12 weeks); Mof, mid-overfeeding (13 weeks); Eof, end overfeeding.
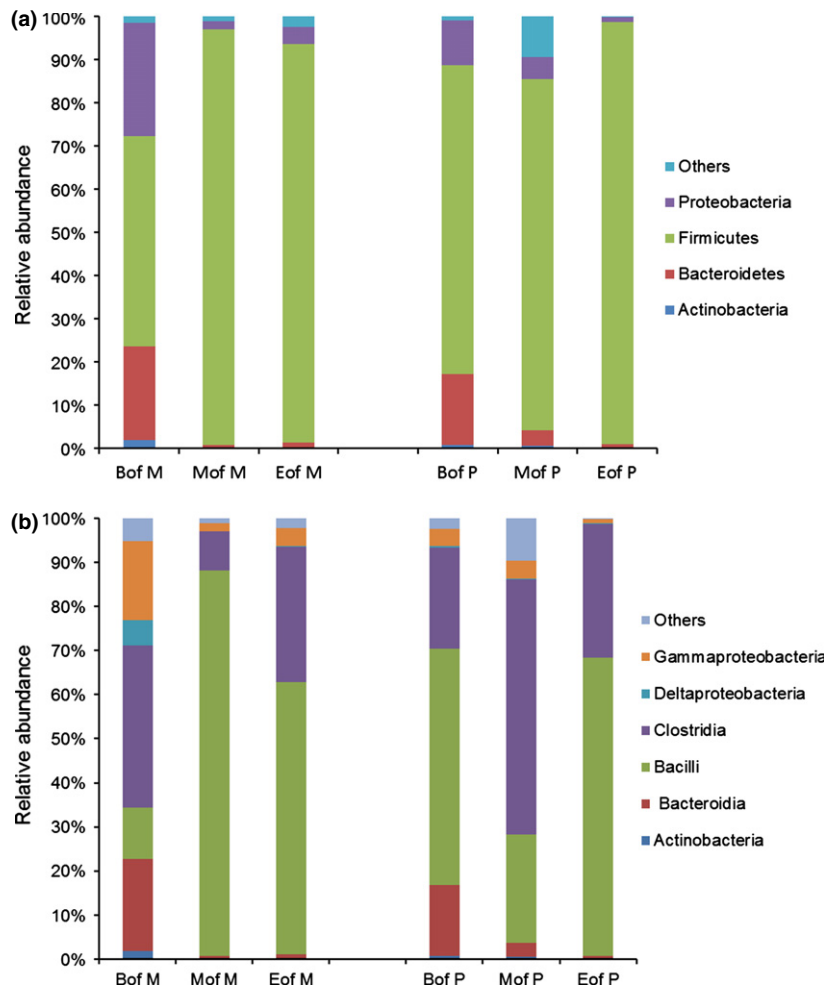*Standard error of the mean.

Fig. 1. Percentage distribution of sequences (%) evaluated at the phylum (a) and class (b) levels to the total number of sequences in the ileum of Muscovy (M) and Pekin (P) ducks during overfeeding (Bof, before overfeeding (12 weeks); Mof, mid-overfeeding (13 weeks); Eof, end of overfeeding).

the community and were not present in all samples (Fig. 2a). At the class level, the *Bacteroidetes* was dominated by *Bacteroidia* (64.3% in Pekin and 50.1% in Muscovy) and *Clostridia* (23.1% in Pekin and 37.1%). *Bacilli* sequences accounted for 4.2% and 2.6% from the *Firmicutes* phyla and those from *Deltaproteobacteria* for 1.4% and 5.8%, in Pekin and Muscovy duck samples, respectively. *Gammaproteobacteria* (1.2%) and *Actinobacteria* were detected only in Pekin duck samples (Fig. 2b). Sequences from the other phyla accounted for < 2% of the community (Fig. 2a). Finally, in Pekin and Muscovy cecal samples, 88 and 113 genera were detected, respectively.

Effect of overfeeding on the ileal and cecal microbial community in Pekin and Muscovy ducks

Overfeeding reduced Chao1 richness, the ACE, and the Shannon index in ileal samples, whereas these different

richness or diversity estimators increased in cecal samples regardless of the genotype of ducks, but with variation (Table 2).

Effect on ileum

Furthermore, the ratio *Firmicutes*/*Bacteroidetes* in ileal samples tended to increase during overfeeding, but with high variation (Bof = 43.8 ± 20.7, Mof = 3082.5 ± 1589.5, Eof = 507.6 ± 444.7) in Pekin and (Bof = 156.1 ± 92.1, Mof = 471.8 ± 337.2, Eof = 408.7 ± 321.9) in Muscovy ducks (Fig. 7). After the different level of intake (overfeeding or not) and the length of the overfeeding period (1 or 2 weeks), the comparison of bacterial communities was analyzed by nMDS profiles (representing the distribution of different families). Ileal sampling distinguished two separate clusters: overfed ducks were found in one cluster and the not-overfed ones in others regardless of the length of overfeeding or genotype of the animals (Fig. 3). The ANOSIM *R*-value indicated the extent to which the
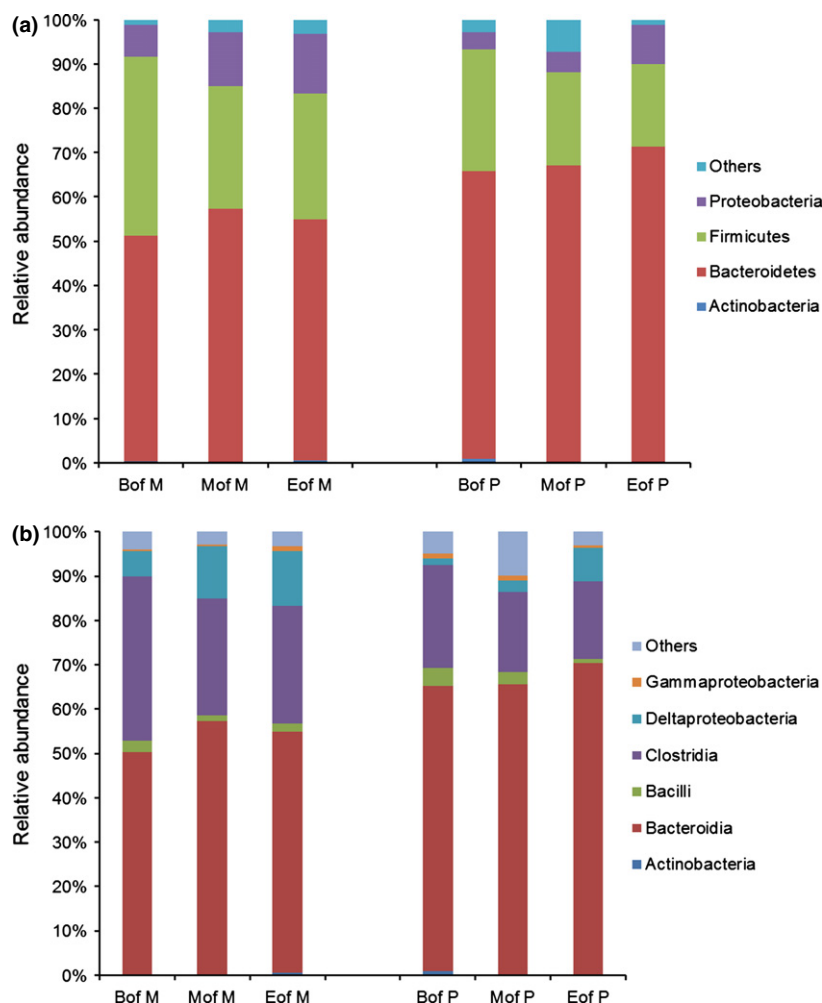
Fig. 2. Percentage distribution of sequences (%) evaluated at the phylum (a) and class (b) levels to the total number of sequences in the ceca of Muscovy (M) and Pekin (P) ducks during overfeeding (Bof, before overfeeding (12 weeks); Mof, mid-overfeeding (13 weeks); Eof, end of overfeeding).

groups differed. For ileal samples, two separate but overlapping groups were observed: R ANOSIM = 0.575 and a $P < 0.001$, which indicates an effect of overfeeding on the bacterial community. Furthermore, there was also an effect of the overfeeding period, namely a statistical difference between the first period (at 12 weeks or before overfeeding) and the last one (at 14 weeks). Two separated but strongly overlapping groups were obtained with R ANOSIM = 0.419 and a $P = 0.009$. This statistical difference was only observable between these two periods (data not shown). The separation of samples by the level of intake was confirmed using the heat map method (Fig. 4). The different effects observed were due to changes in different families such as *Leuconostocaceae* for the genotype effect or mainly *Lactobacillaceae* or *Streptococcaceae* for the period effect. All statistical changes (P-value < 0.05) are listed in Table 3, and there were no interactions. For each sample, the diversity of all families detected has been observed; families with the largest diversity were *Clostridiaceae*, *Lactobacillaceae*, *Ruminococcaceae*, and
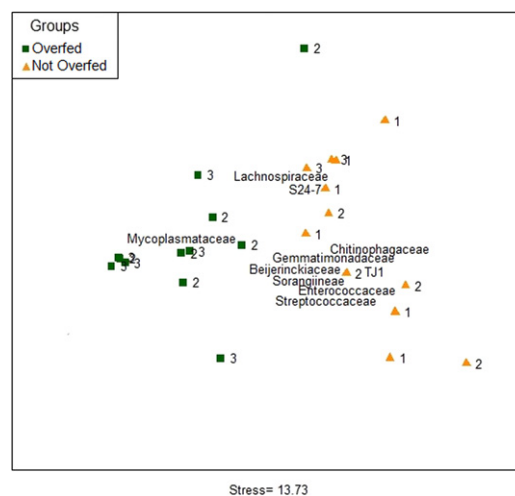

Fig. 3. nMDS profile showing the distribution of different families of the different samples studied overfed (■) or not-overfed (▲) in three periods of overfeeding in the ileum. Before overfeeding (1), mid-overfeeding (2), and end (3) of overfeeding.

Fig. 4. Heat map of the bacterial community at the family levels of Muscovy (M) and Pekin (P) for overfed (OF) or not-overfed (NOF) ducks in the ileum. Samples and taxa were clustered according to Ward algorithm based on a Manhattan distance matrix. Before overfeeding (1), mid-overfeeding (2), and end (3) of overfeeding.

Table 3. Statistical effect of the genotype and period on different families with a significant *P*-value (< 0.05) in the ileum of Muscovy and Pekin ducks

		Muscovy		Pekin	
	P-value	Mean	SEM*	Mean	SEM*
Genotype effect					
Leuconostocaceae	0.028	0.45	0.17	0.14	0.07
Feeding period effect					
Enterococcaceae	0.003	0.70	0.57	13.08	6.93
Staphylococcaceae	0.009	0.05	0.02	0.05	0.02
Actinomycetales	0.014	0.31	0.16	0.48	0.18
Lactobacillaceae	0.016	38.79	12.18	23.20	10.39
Carnobacteriaceae	0.024	0.03	0.01	0.08	0.04
Streptococcaceae	0.033	14.61	8.91	4.72	2.23

*Standard error of the mean.

Streptococcaceae, which played an important role in the clustering (Fig. 4).

Effect on ceca

In ceca samples, the ratio *Firmicutes/Bacteroidetes* (Bof = 0.4 ± 0.1, Mof = 0.3 ± 0.1, Eof = 0.3 ± 0.1) was stable in Pekin ducks during overfeeding, whereas the richness estimators increased. Regarding cecal samples, nMDS profiles also showed that the samples were slightly separated according to the treatment (overfed or not): $R_{\text{ANOSIM}} = 0.116$ and $P = 0.010$ (Fig. 5a). As in ileal samples, the heat map method revealed the separation by genotype and level of intake (Fig. 6). The *Firmicutes/Bacteroidetes* ratio in Muscovy ducks tended to decrease at mid-overfeeding and increase at the end of overfeeding (Bof = 0.8 ± 0.1, Mof = 0.5 ± 0.1, Eof = 0.9 ± 0.6; Fig. 7). A significant difference ($P < 0.05$) was observed between the two genotypes for the *Firmicutes/Bacteroidetes* ratio. Several families were responsible for these effects especially *Bacteroidaceae*, *Desulfovibrionaceae*, and *Lachnospiraceae* for the genotype effect or *Streptococcaceae* for the overfeeding effect. All changes for these families were significant ($P < 0.05$) and are listed in Table 4. In our samples, seven families showed a high diversity: *Lachnospiraceae*, *Porphyromonadaceae*, *Rikenellaceae*, *Prevotellaceae*, *Ruminococcaceae*, and *Bacteroidaceae*. Interestingly, there was also a genotype effect between Pekin and Muscovy ducks regardless of age or the period of overfeeding, with a few separations in to groups: $R_{\text{ANOSIM}} = 0.195$ and a $P = 0.001$ (Figs 5b and 6).

Discussion

In this study, high-throughput sequencing was used for the first time to identity the microbial diversity of ileal and cecal samples from ducks, after which the effect of
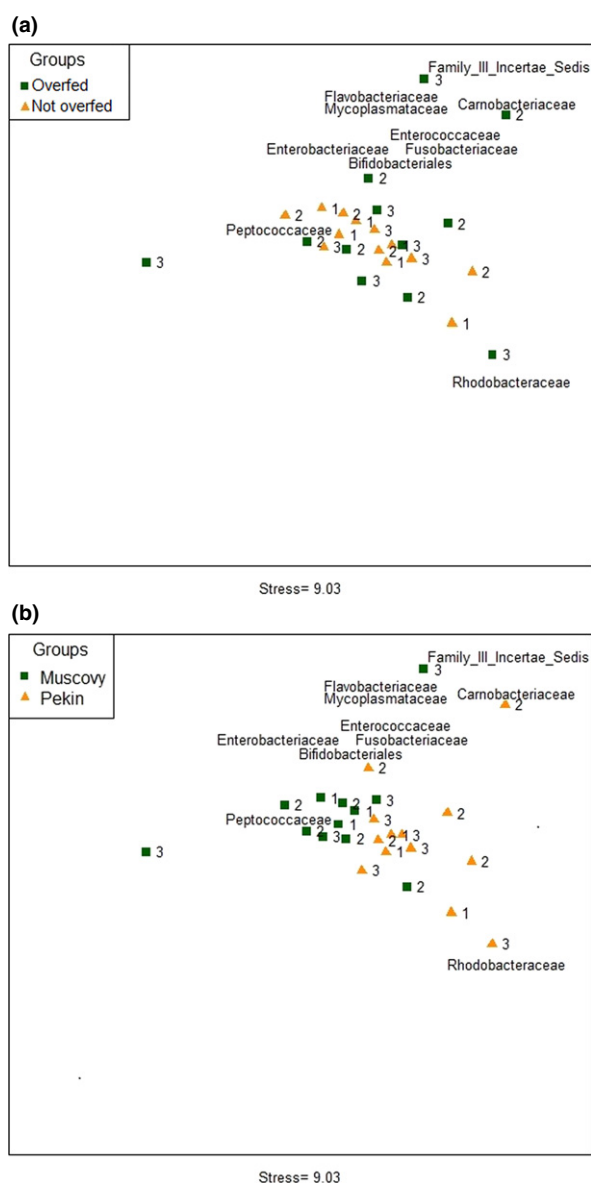

Fig. 5. nMDS profile showing the distribution of different families of the different samples studied (a) overfed (■) or not-overfed (▲) and (b) Muscovy (■) or Pekin (▲) ducks in three periods of overfeeding in the ceca. Before overfeeding (1), mid-overfeeding (2), and end (3) of overfeeding.

overfeeding or genetics on that diversity was studied. At the phylum level, *Firmicutes* and *Bacteroidetes* (more than 80% of sequences) were major bacteria in the duck gut microbiota. Most studies on microbiota have shown that *Firmicutes* and *Bacteroidetes* are the two most dominant phyla in different organisms such as birds or mammals suggesting an important conserved role in intestinal microbiota metabolism (Ley *et al.*, 2008; Kohl, 2012). Costello *et al.* (2010) suggest that the common ancestor of amniotes (birds, reptiles, and mammals) harbored a

Fig. 6. Heat map of the bacterial community at the family levels of Muscovy (M) and Pekin (P) for overfed (OF) or not-overfed (NOF) ducks in the ceca. Samples and taxa were clustered according to a Ward algorithm based on a Manhattan distance matrix. Before overfeeding (1), mid-overfeeding (2), and end (3) of overfeeding.

Table 4. Statistical effect of the genotype and period on different families with a significant *P*-value (*P* < 0.05) in the ceca of Muscovy and Pekin ducks

		Muscovy		Pekin	
	P-value	Mean	SEM*	Mean	SEM*
Genotype effect					
Deferribacteraceae	0	0.06	0.03	0.34	0.09
Succinivibrionaceae	0	0.07	0.03	0.74	0.22
Peptococcaceae	0.003	0.15	0.05	0.03	0.02
Lachnospiraceae	0.005	9.67	1.44	4.89	0.65
Family_XIII_Incertae_Sedis	0.006	0.30	0.07	0.09	0.02
Alcaligenaceae	0.008	0.08	0.04	0.39	0.10
Clostridiaceae	0.01	0.01	0.01	0.17	0.09
Bacteroidaceae	0.01	25.12	2.73	37.08	3.21
Desulfovibrionaceae	0.019	8.94	2.07	4.66	1.09
Fusobacteriaceae	0.037	0.08	0.04	1.80	1.35
Feeding period effect					
Comamonadaceae	0.001	0.02	0.01	0.02	0.01
Streptococcaceae	0.004	1.85	0.78	0.57	0.28
Anaeroplasmataceae	0.023	0.03	0.01	0.05	0.02

*Standard error of the mean.

microbiota composed essentially of *Firmicutes* and *Bacteroidetes*. Furthermore, as previously described in chickens, ceca were dominated by obligate anaerobes (*Bacteroidetes* including *Bacteroidia* and *Firmicutes* including *Clostridia*) in both Pekin and Muscovy ducks, but differences occur with chickens. In this study, cecal segments were dominated by *Bacteroidetes* in ducks, whereas *Firmicutes* (especially the class *Clostridia*) is the major phylum in chickens (Zhu *et al.*, 2002; Lu *et al.*, 2003). Interestingly, turkeys also show *Bacteroidetes* as the major phylum in cecum regardless of the phylogenetic distance between turkey and ducks, suggesting that environmental conditions and diet could play a more important role than genetics. Furthermore, although *Firmicutes* were dominant in ileal segments in chickens and ducks, interesting changes have been identified at the class and family levels (Barnes, 1979; Mead, 1989; Lu *et al.*, 2003). In chickens and Pekin ducks, sequences related to the *Bacilli* order (from *Firmicutes*) were dominant (80% for chickens and 51% for ducks), but the family distribution was quite different. In Pekin ducks, the genera *Streptococcus* spp. and *Enterococcus* spp. represented most of the *Bacilli* sequences (50%), while in chickens, around 70% of sequences are related to the *Lactobacillus* genus (Lu *et al.*, 2008). Regarding Muscovy ducks, the bacterial community in the ileum was not dominated by facultative anaerobes, but by obligate anaerobes such as *Clostridia* and *Bacteroidia*. In humans, the ratio *Firmicutes*/*Bacteroidetes* is closely related to weight; it increases in obese people and may decrease during weight loss (Ley *et al.*, 2006). In this study, it tended to increase in the ileal segment, but

with high variation (Fig. 7). The *Firmicutes*/*Bacteroidetes* ratio appeared to be correlated with overfeeding in the ileum and with genotype in the ceca. Overfeeding in ducks promotes an important hepatic steatosis induced by an increased rate of lipogenesis from carbohydrates and a defect in hepatic lipid secretion (Saadoun & Leclercq, 1987; Fournier *et al.*, 1997). It is well known that overfed Pekin ducks have a higher lipid content in peripheral tissues (muscle and adipose tissues), while liver storage is lower than in other species (Davail *et al.*, 2003a; Chartrin *et al.*, 2006). Otherwise, the LPL activity in ducks correlates positively with a higher storage in peripheral tissues instead of fat storage in the liver (Saez *et al.*, 2010). Interestingly, in mammals, the microbiota triggers the storage of triglycerides through suppression of the intestinal expression of fasting-induced adipocyte factor, a circulating LPL inhibitor (Backhed *et al.*, 2004). Could these changes in the microbial community during overfeeding affect a predisposition to liver or peripheral fat storage in ducks? In our study, overfeeding reduced the bacterial diversity estimated by the Shannon index in ileal samples. Overfeeding affected the relative abundance of *Firmicutes* and especially some genera from the *Bacilli* class (*Lactobacillaceae*, *Enterococcaceae*, and *Streptococcaceae*) regardless the genotype. The relative abundance of *Lactobacillaceae* spp. increased strongly with overfeeding in both Pekin and Muscovy ducks leading to a decrease in most other families. These bacteria are well known as amylolytic and lactate-producing bacteria and frequently increase in animals (e.g. pigs and rats) fed with diets rich in starch (Wang *et al.*, 2002; Regmi *et al.*, 2011). In cattle, it has been shown that *Lactobacillus* spp. increase with high-concentrate diets containing more than 70% of starch (Brown *et al.*, 2006). Furthermore, *Streptococcaceae*, another amylolytic bacterial family, is decreased by overfeeding in Pekin and Muscovy ducks, whereas in cows, Fernando *et al.* (2010) report the increase in *Streptococcus* spp. with a high grain diet and especially *Streptococcus bovis*. In birds, the effect of diets on the abundance of *Enterococcus* spp. and *Streptococcus* spp. has not yet been published. Another member of *Bacilli*, the *Enterococcaceae* family, was affected, but only in Pekin ducks. Very little is known in birds regarding the effect of diets on these different families. A very small effect of the genotype was also detected in the *Leuconostocaceae* family, from the class *Bacilli*, a new family identified in 2010 (Schleifer, 2010). Finally, regarding ileal samples, regardless of the genotype and the length of the diet, they were only separated by the diet type (overfed or not), and the most affected bacterial groups were the *Lactobacillaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Streptococcaceae*, and *Ruminococcaceae* (Fig. 4 or Fig. 3). Interestingly, in cecal samples, the effect of the genotype was more
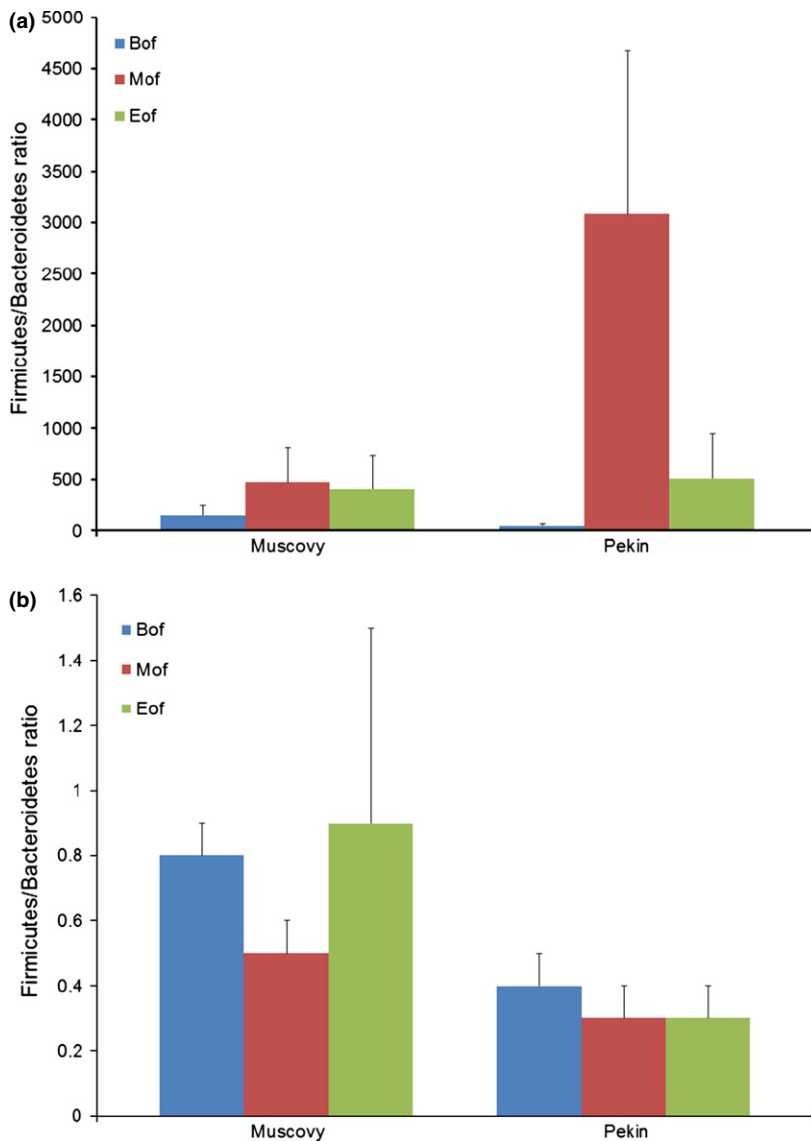
Fig. 7. *Firmicutes/Bacteroidetes* ratio of Muscovy and Pekin ducks during overfeeding (before overfeeding: Bof, mid-overfeeding: Mof, and end of overfeeding: Eof) in ileum (a) and ceca (b).

important than the effect of overfeeding, suggesting that the complexity and the anaerobic environment of the ceca did not allow important changes in the bacterial community. Furthermore, it has been suggested that transit times are longer in the ceca, permitting a better microbial fermentation (Rehman *et al.*, 2007). The physiology of Pekin and Muscovy ducks is quite different as previously described, and several metabolites such as short-chain fatty acids (SCFA) or amino acids from the ceca could be a part of that difference (Józefiak *et al.*, 2004). Three families show significant differences between Pekin and Muscovy ducks: *Lachnospiraceae*, *Bacteroidaceae*, and *Desulfovibrionaceae* from three different phyla. Moreover, *Lachnospiraceae* from *Firmicutes* and *Bacteroidaceae* are two families with bacteria with amylolytic and cellulolytic properties (Cotta, 1987; Wedekind *et al.*, 1988; Flint *et al.*, 2012).

Regarding *Desulfovibrionaceae*, some family members can produce acetate (one well known SCFA) and are among the sulfate-reducing bacteria (Devereux *et al.*, 1990). There was also a need to find an effect of overfeeding on three families in both Pekin and Muscovy ducks: *Streptococcaceae* in ileal samples and two poorly documented families, *Comamonadaceae* (from *Proteobacteria*) and *Coriobacteriales* (wall-less bacteria belonging to *Firmicutes* and the *Mollicutes* class). Finally, in cecal samples, the bacterial community could be separated by both the genotype (Fig. 5a) and the level of intake (Fig. 5b), but the former had the greater effect. Heat maps confirmed these results, and the most affected bacterial groups were the families described above (Fig. 6). The genetic effect on microbiota composition was also well described in mice (Friswell *et al.*, 2010; Kovacs *et al.*, 2010).

In conclusion, this study using a high-throughput pyrosequencing based on the 16S rRNA gene in ileal and cecal samples from ducks provides a first inventory of the microbial community and the effect of overfeeding and genotype on the abundance of major different groups. Overfeeding affected the richness diversity of ileal and cecal samples and had a significant effect in modifying the bacterial community in the ileum, whereas genotype mainly affected the ceca. The microbial diversity of ducks' microbiota was dominated by *Firmicutes* and *Bacteroidetes*. Further investigations are necessary to understand the functional microbiota in ducks and enhance the digestibility of resistant starch during overfeeding periods in ducks. This would be of great interest for researchers studying intestinal microbiota in poultry, and this new knowledge could be used to prepare birds to overfeeding.

Acknowledgements

References

Ahmed N, Qu A, Brulc JM *et al.* (2008) Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS ONE* **3**: e2945.

Andersson AF, Lindberg M, Jakobson H, Bäckhed F, Nyrén P & Engstrand L (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **3**: e2836.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF & Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. *P Natl Acad Sci USA* **101**: 15718–15723.

Barnes E (1979) The intestinal microflora of poultry and game birds during life and after storage. *J Appl Bacteriol* **46**: 407–419.

Brown MS, Ponce CH & Pulikanti R (2006) Adaptation of beef cattle to high-concentrate diets: performance and ruminal metabolism. *J Anim Sci* **84**: E25–E33.

Chartrin P, Bernadet M-D, Guy G *et al.* (2006) Does overfeeding enhance genotype effects on energy metabolism and lipid deposition in breast muscle of ducks? *Comp Biochem Physiol A: Mol Integr Physiol* **145**: 413–418.

Costello EK, Gordon JI, Secor SM & Knight R (2010) Postprandial remodeling of the gut microbiota in Burmese pythons. *ISME J* **4**: 1375–1385.

Cotta MA (1987) Amylolytic activity of selected species of ruminal bacteria. *Appl Environ Microbiol* **54**: 772–776.

Davail S, Rideau N, Guy G, André J-M, Hermier D & Hoo-Paris R (2003a) Hormonal and metabolic responses to overfeeding in three genotypes of ducks. *Comp Biochem Physiol A: Mol Integr Physiol* **134**: 707–715.

Davail S, Rideau N, Guy G, André JM & Hoo-Paris R (2003b) Pancreatic hormonal and metabolic responses in overfed ducks. *Horm Metab Res* **35**: 439–443.

Devereux R, Shao-Hua H, Doyle CL, Orkland S, Stahl DA, LeFall J & Whitman WB (1990) Diversity and origin of Desulfovibrio species: phylogenetic definition of a family. *J Bacteriol* **172**: 3609–3619.

Fernando SC, Purvis HT, Najar FZ *et al.* (2010) Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl Environ Microbiol* **76**: 7482–7490.

Flint HJ, Scott KP, Duncan SH, Louis P & Forano E (2012) Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**: 289–306.

Fournier E, Peresson R, Guy G & Hermier D (1997) Relationships between storage and secretion of hepatic lipids in two breeds of geese with different susceptibility to liver steatosis. *Poult Sci* **76**: 599–607.

Friswell MK, Gika H, Stratford IJ, Theodoridis G, Telfer B, Wilson ID & McBain AJ (2010) Site and strain-specific variation in gut microbiota profiles and metabolism in experimental mice. *PLoS ONE* **5**: e8584.

Fuller R (1984) Microbial activity in the alimentary tract of birds. *Proc Nutr Soc* **43**: 55–61.

Gordon H & Pesti L (1971) The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev* **35**: 390–429.

Hayek LC & Buzas MA (1996) *Surveying Natural Populations.* Columbia University Press, New York, NY.

Hermier D, Guy G, Guillaumin S, Davail S, André J-M & Hoo-Paris R (2003) Differential channelling of liver lipids in relation to susceptibility to hepatic steatosis in two species of ducks. *Comp Biochem Physiol B: Biochem Mol Biol* **135**: 663–675.

Johansen CH, Bjerrum L & Pedersen K (2007) Impact of salinomycin on the intestinal microflora of broiler chickens. *Acta Vet Scand* **49**: 30.

Józefiak D, Rutkowski A & Martin SA (2004) Carbohydrate fermentation in the avian ceca: a review. *Anim Feed Sci Technol* **113**: 1–15.

Kohl KD (2012) Diversity and function of the avian gut microbiota. *J Comp Physiol B* **182**: 591–602.

Kovacs A, Ben-Jacob N, Tayem H, Halperin E, Iraqi FA & Gophna U (2010) Genotype is a stronger determinant than sex of the mouse gut microbiota. *Microb Ecol* **61**: 423–428.

Ley RE, Tumbaugh PJ, Klein S & Gordon JI (2006) Human gut microbes associated with obesity. *Nature* **444**: 1022–1023.

Ley RE, Hamady M, Lozupone C *et al.* (2008) Evolution of mammals and their gut microbes. *Science* **320**: 1647–1651.

Liu Z, Lozupone C, Hamady M, Bushman FD & Knight R (2007) Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Res* **35**: e120.

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

Lu J, Hofacre C, Smith F & Lee MD (2008) Effects of feed additives on the development on the ileal bacterial community of the broiler chicken. *Animal* **2**: 669–676.

Mead G (1989) Microbes of the avian cecum: types present and substrates utilized. *J Exp Zool* **3**: 48–54.

Mead GC & Adams BW (1975) Some observations on the caecal microflora of the chick during the first two weeks of life. *Br Poult Sci* **16**: 169–176.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Regmi PR, Metzler-Zebeli BU, Ganzle MG, van Kempen TATG & Zijlstra RT (2011) Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes Bifidobacteria in pigs. *J Nutr* **141**: 1273–1280.

Rehman H, Vahjen W, Awad WA & Zentek J (2007) Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. *Arch Anim Nutr* **61**: 319–335.

Saadoun A & Leclercq B (1987) *In vivo* lipogenesis of genetically lean and fat chickens: effects of nutritional state and dietary fat. *J Nutr* **117**: 428–435.

Saez G, Baeza E, Bernadet MD & Davail S (2010) Is there a relationship between the kinetics of lipoprotein lipase activity after a meal and the susceptibility to hepatic steatosis development in ducks? *Poult Sci* **89**: 2453–2460.

Schleifer KH (2010) Family V. Leuconostocaceae fam. nov. *Bergey's Manual of Systematic Bacteriology*, Vol. 3 (The Firmicutes), 2nd edn (De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH & Whitman WB, eds), pp. 624. Springer, Dordrecht, Heidelberg, London, New York.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**: 7537–7541.

Serino M, Luche E, Gres S *et al.* (2011) Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut* **61**: 543–553.

Taschuk R & Griebel PJ (2012) Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. *Anim Health Res Rev* **13**: 129–141.

Wang X, Brown IL, Khaled D, Mahoney MC, Evans AJ & Conway PL (2002) Manipulation of colonic bacteria and volatile fatty acid production by dietary high amylose maize (amylomaize) starch granules. *J Appl Microbiol* **93**: 390–397.

Wedekind KJ, Mansfield HR & Montgomery L (1988) Enumeration and isolation of cellulolytic and hemicellulolytic bacteria from human feces. *Appl Environ Microbiol* **54**: 1530–1535.

Zhu XY, Zhong T, Pandya Y & Joerger RD (2002) 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Appl Environ Microbiol* **68**: 124–137.