Effect of a low protein diet on chicken ceca microbiome and productive performances

Alessandra De Cesare,[*] Ìtalo Faria do Valle,[†,‡] Claudia Sala [ID],[†,1] Federico Sirri,[*] Annalisa Astolfi,[§] Gastone Castellani,[†,#] and Gerardo Manfreda[*]

[*]*Department of Agricultural and Food Sciences, University of Bologna, 40126, Bologna, Italy;* [†]*Department of Physics and Astronomy, University of Bologna, 40126, Bologna, Italy;* [‡]*CAPES Foundation, Ministry of Education of Brazil, 70040-020, Brasìlia, DF, Brazil;* [§]*Centro Interdipartimentale di Ricerche sul Cancro "Giorgio Prodi" (CIRC), 40126, Bologna, Italy; and* [#]*Centro Interdipartimentale "L. Galvani" per Studi Integrati di Bioinformatica, Biofisica, Biocomplessità (CIG), 40126, Bologna, Italy*

ABSTRACT The aim of this study was to investigate the impact of supplementation of a low protein diet on ceca microbiome and productive performances of broiler chickens. A total of 1,170 one-day-old male chicks (Ross 308) were divided in 2 diet groups and reared in the same conditions up to 42 D. Birds belonging to the control group were fed a basal diet. Birds belonging to the low protein group the basal diet with a reduced level of crude protein (–7%). Cecum contents from randomly selected birds were collected at 14 and 42 D within each diet group, submitted to DNA extraction and then tested by shotgun metagenomic sequencing. Abundances of species belonging to Actinobacteria and Proteobacteria were mainly affected by the diet as well as interaction between diet and time, while species belonging to Firmicutes and Cyanobacteria changed mainly according to the age of the birds. At family level, Lactobacillaceae significantly decreased in the low protein group up to 14 D. However, at the end of the rearing period the same family was significantly higher in the low protein group. The most abundant functional genes, represented by cystine desulfurase, alpha-galactosidase, and serine hydroxymethyltransferase, displayed comparable abundances in both diet groups, although significative differences were identified for less abundant functional genes at both sampling times. Birds fed control and low protein diets showed similar productive performances. However, in the finisher phase, feed conversion rate was significantly better in chickens fed the low protein diet. Overall, this study showed that a reduced intake of crude protein in broilers increases the abundance of Lactobacillaceae in the ceca over time and this seems to be linked to a better feed conversion rate between 36 and 42 D. A reduced intake of crude protein in chicken production can help to improve exploitation of edible resources, while reducing the emission of nitrogen pollutants in the environment.

Key words: low protein diet, chickens, metagenomics, microbiome

INTRODUCTION

Chickens are a high-quality protein source for humans. Like other birds, they have proportionally smaller intestines and shorter transit digestion times than mammals (McWhorter et al., 2009). In chicken, gastrointestinal tract (**GIT**) microbiota and its metabolites play a central role in enhancing nutrient absorption and strengthening the immune system. GIT microbiota comprises a diverse collection of microbial species. For many years, such specie have been investigated by culture-dependent methods (Barnes et al., 1972; Salanitro et al., 1974). However, it is estimated that only 10 to 60% of cecal microorganisms can be cultivated and only 45% of chicken intestinal bacteria can be assigned to a known genus (Apajalahti et al., 2004). Therefore, much of GIT microbiota remains largely unexplored, making it a major source of untapped biological potential in terms of newly identified bacteria, encoded enzyme activities, and potentially probiotic strains (Stanley et al., 2014).

In recent years, molecular technology is moving towards high-throughput next-generation sequencing (**HT-NGS**), providing large-scale analysis with unprecedented depths and coverage. Among HT-NGS technologies, shotgun metagenomic sequencing has dramatically expanded our knowledge on microbiota composition in various environments, including chicken gut. Fully enumerating of all the DNA sequences carried by each organism in a given environment is still impossible (Ni et al., 2013). However, metagenomic results

can contribute to clarify key biological questions, like how microbiota stability and ecological shifts are influenced by nutrients and hosts (Andersson et al., 2008). In particular, the key issue in animal production, including chicken nutrition, is to understand the relationship among effects of diet composition and changes in microbiota and host metabolism.

Protein is considered as one of the most expensive nutrient in commercial poultry feed. Reduction of dietary protein level and use of synthetic amino acid are crucial to minimize the feed cost and to contain nitrogen emission, thus limiting environmental pollution. Moreover, there are several evidences that a considerable amount of protein and amino acids pass through the gut and are excreted as partially or completely undigested fractions (Parson et al., 1997; Lemme et al., 2004). Although modern broiler chickens represent a major animal husbandry success story, in terms of both efficient resource utilization and environmental sustainability (Tallentire et al., 2018), conversion of dietary nitrogen to animal products is relatively inefficient. In fact, broiler and commercial layer, respectively, excrete 50 to 65% of dietary nitrogen, affecting air and surface ground water quality (Chalova et al., 2016). Even though nitrogen may exist in various forms, ammonia is of particular importance because of its potential to volatilize and negatively impact water and air quality (Sommer and Hutchings, 2001). Therefore, nitrogen overloading of poultry diets is still a challenge that poses a risk for the environment as well as animal and human health. Intensive production systems consume large amounts of protein and other nitrogen containing substances in animal feed. Ferket et al. (2002) demonstrated that nitrogen excretion can be significantly reduced by lowering dietary crude protein levels and optimizing the supplementation of synthetic amino acids.

Composition and digestibility of the diet deeply influence the intestinal microbiota, since compounds of dietary origin are the most important growth substrates for microbes. Diets are formulated to match nutritional requirements of the chickens, even though management of the highly active microbiota through the diet may also play an important role in host's health and performances (Apajalahti and Vienola, 2016). It is well known that nutritional requirements of bacteria are species-specific. For instance, *Lactobacillus*, which dominate the small intestine of chicken, have very high complex nutrient requirements and are unable to grow if amino acids, vitamins, and many other compounds are not available (Morishita et al., 1981). Conversely, *Escherichia coli*, also present in the small intestine, is independent of exogenous amino acids, requiring only sugar and minerals. Like *Lactobacillus*, *Clostridium perfringens*, the causative agent of necrotic enteritis, is also dependent on several amino acids in the habitat in which it resides (Wilkie et al., 2005).

Considering the effects exerted by diet nitrogen compounds on growth of bacteria living in the chicken gut, in this study, taxonomic and functional changes occurring at 14 and 42 D in the microbiome colonizing ceca of chickens fed a diet with a reduced level of crude protein (–7%) were investigated in comparison to a control group by shotgun metagenomic sequencing. The impact of the low protein diet on productive performances was assessed as well.

MATERIALS AND METHODS

Birds were handled according to the principles stated in the EU Directive 63/2010 (European Commission, 2010) regarding the protection of animals used for experimental and other scientific purposes and according to the guidelines of the Animal Ethic Committee of the University of Bologna. A permit number is not reported because it was provided only for trials performed after the Directive 2010/63/EU took full effect (i.e., 2014 March 14). Birds were farmed and slaughtered under commercial conditions, according to Italian and European law for protection of chicken kept for meat production (European Commission, 2007). At 14 and 42 D, 10 birds/sampling time were humanely euthanized by cervical dislocation for the ceca collection. According to the Council Regulation (EC) No. 1099/2009 (European Commission, 2009) on protection of animals at the time of killing, the remaining chickens were electrically stunned and slaughtered. All slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health.

Animals and Diet Groups

A total of 1,170 one-day-old male chicks (Ross 308) were obtained from a commercial hatchery. All chicks belonged to the same breeder flock and hatching session. The chicks were housed in September 2013 in a poultry house containing 18 pens of 6 m² each. Before housing, birds were individually weighed and divided according to their live weight in 3 classes: 42 to 44, 45 to 47, 48 to 50 g. The groups were distributed in 18 pens at the stocking density of about 10 chicks/m² (i.e., 65 birds/pen), while maintaining the same class distribution of live weight of the population. The 18 pens were divided in 2 experimental groups of 9 replicates each. Pens labeled as group A (control) hosted birds fed a corn-soybean basal diet formulated according to the Ross 308 nutrient specifications; those of group B (low protein) birds fed with basal diet –7% crude protein. The analyzed composition of each experimental diet is provided in Table 1. The pens were distributed in a randomized complete block design to minimize the effects of the environment. Feeds were supplied ad libitum in mash form throughout the experiment. The feeding program included 4 phases: starter (0 to 11 D), grower first period (12 to 21 D), grower second period (21 to 35 D), and finisher (36 to 42 D). The experiment lasted 42 D, when birds reached the slaughter weight of about 2.8 kg of live weight. Photoperiod and temperature

Table 1. Composition of the experimental diets in each feeding phase[1].

Feeding phases	Pre-starter (0 to 11 D)		Starter (12 to 20 D)		Grower (21 to 35 D)		Finisher (36 to 42 D)	
Experimental groups	A	B	A	B	A	B	A	B
Ingredients (g/kg)								
Corn	40.0	43.9	46.2	49.9	47.9	51.5	48.6	52.3
Wheat	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Soybean, extracted meal	25.0	21.7	20.1	16.9	19.9	16.8	19.3	16.1
Expanded full-fat soybean	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Corn gluten meal	3.0	3.0	2.0	2.0	0.0	0.0	0.0	0.0
Vegetable oil	2.6	2.0	3.1	2.5	4.3	3.7	4.5	4.0
Dicalcium phosphate	1.5	1.6	1.2	1.3	0.8	0.9	0.6	0.6
Calcium carbonate	1.0	1.0	0.7	0.7	0.6	0.6	0.6	0.6
Salt	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2
Sodium bicarbonate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Coline chloride	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Lysine sulphate	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.3
DL-methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Threonine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phytase	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin-mineral premix	0.4	0.4	0.4	0.4	0.3	0.3	0.2	0.2
Calculated composition (g/kg)								
Dry matter*	88.6	88.5	88.5	88.6	88.4	88.2	88.5	88.2
Protein*	22.8	21.2	20.5	19.5	19.8	18.0	19.2	17.9
Lipid*	6.45	6.21	7.55	6.91	8.45	7.98	8.65	8.44
Fiber*	2.64	2.59	2.59	2.53	2.57	2.52	2.56	2.05
Ash*	5.27	5.14	5.07	4.94	4.21	4.85	4.44	4.01
Total lysine*	1.36	1.27	1.21	1.12	1.14	1.06	1.11	1.03
Total methionine	0.66	0.61	0.60	0.56	0.58	0.53	0.56	0.52
Total methionine+cystine	1.02	0.96	0.93	0.87	0.89	0.83	0.87	0.81
Ca	0.91	0.91	0.79	0.79	0.64	0.64	0.56	0.56
P	0.62	0.62	0.55	0.55	0.47	0.47	0.43	0.43
ME (Kcal/kg)	3,070	3,070	3,160	3,160	3,240	3,240	3,275	3,275

[1]Provided the following per kg of diet: vitamin A (retinyl acetate), 13,000 IU; vitamin D3 (cholecalciferol), 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B$_{12}$ 20 μg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; ethoxyquin, 100 mg.

*Analyzed composition.

programs were set up according to the European Welfare Regulation 43/2007 (European Commission, 2007). At 0, 11, 21, 35, and 42 D (slaughter), birds were counted and weighed on a pen basis. At the same intervals, feed orts were weighed pen wise. Daily weight gain (**DWG**), daily feed intake (**DFI**), feed conversion rate (**FCR**), and mortality were calculated for each feeding phase and for the overall experimental period. DFI and FCR were corrected for mortality, taking into account weight and life duration of dead birds.

Sample Collection

To characterize the impact of each experimental diet on broiler ceca microbiome, 5 chickens were randomly selected and humanely euthanized at 14 and 42 D from each diet group (i.e., A and B). The entire GIT of each individual selected bird was dissected out and a small portion (i.e., 0.5 to 2 g) of cecum content was collected from both ceca into 2 mL sterile plastic tubes. The 20 collected tubes were then stored at –80°C until DNA extraction.

DNA Extraction

The DNA was extracted from each sample using a bead-beating procedure (Danzeisen et al., 2011). Briefly, 0.25 g of cecal content were suspended in 1 mL lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, 4% SDS) with MagNA Lyser Green Beads (Roche, Milan, Italy) and homogenized on the MagNA Lyser (Roche) for 25 s at 6,500 rpm. The samples were then heated at 70°C for 15 min, followed by centrifugation to separate the DNA from the bacterial cellular debris. This process was repeated with a second 300 μL aliquot of lysis buffer. The samples were then subjected to 10 M v/v ammonium acetate (Sigma, Milan, Italy) precipitation, followed by isopropanol (Sigma) precipitation and a 70% ethanol (Carlo Erba, Milan, Italy) wash and resuspended in 100 μL 1X Tris-EDTA (Sigma). The samples were then treated with DNase-free RNase (Roche) and incubated overnight at 4°C, before being processed through the QIAmp DNA Stool Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's directions, with some modifications. Samples were measured on a BioSpectrometer (Eppendorf, Milan, Italy) to assess DNA quantity and quality.

Metagenomic Sequencing

Total DNA from each individual sample was fragmented and tagged with sequencing adapters using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). Shotgun whole genome sequencing was then performed using HiScanSQ sequencer (Illumina) at 100 bp, in paired-end mode. Metagenomic

sequencing yielded an average of 5.98 million reads/sample. Following sequencing, all reads were quality assessed and the paired end was merged.

Metagenomic Sequences Analysis

The metagenomic sequences were deposited in MG-RAST (http://metagenomics.anl.gov/) under project label as "low protein" with the following IDs: group A 14 D mgm 4,624,496.3, 4,624,508.3, 4,624,511.3, 4,624,512.3, 4,624,518.3; group A 42 D mgm 4,624,490.3, 4,624,492.3, 4,624,493.3, 4,624,494.3, 4,624,513.3; group B 14 D mgm 4,624,489.3, 4,624,503.3, 4,624,510.3, 4,624,514.3, 4,624,515.3; group B 42 D mgm 4,624,497.3, 4,624,501.3, 4,624,502.3, 4,624,504.3, 4,624,516.3. After applying the quality control procedure, following the instructions of the MG-RAST manual, the taxonomic classification of the sequencing data was performed by applying the Best Hit Classification method and using the M5RNA database, whereas the functional classifications were performed by using the SEED Subsystems annotations. In both cases, the following parameters were set: maximum e-value 1e-5, minimum identity 60%, and minimum alignment length 15 bp.

Statistical Analysis

After removing non-bacterial species, taxa abundances obtained from MG-RAST were normalized so that each sample total abundance resulted 1. The samples heterogeneity was investigated both in terms of alpha and beta diversity. Alpha diversity was computed according to Pielou's definition (Pielou, 1996):

$$\alpha = \frac{-\sum_{i=1}^{S} p_i ln(p_i)}{ln(S)},$$

where p_i is the proportion of the ith species. This is a normalized index related to Shannon entropy, which ranges from 0, when all individuals belong to the same species, corresponding to minimum diversity, to 1, when each individual belongs to a different species, corresponding to maximum diversity.

Beta diversity, referring to the compositional difference between samples, was computed in the form of Bray–Curtis distance with pdist function of scipy module (0.17.0). Principal coordinate analysis was computed with python module skbio 0.4.2 to represent the samples in a 2D space that satisfies the Bray–Curtis distances. In order to assess which species mostly contribute to the samples Bray-Curtis distances, SIMilarity PERcentages analysis (**SIMPER**) (Clarke, 1993) was computed with R package vegan 2.3–5, setting 100,000 permutations. Species that contribute at least to 70% of the differences between groups were selected by SIMPER as important in ex-

plaining the beta diversities. Student's t-test and 2-way analysis of variance (ANOVA) were computed with scipy 0.17.0, after scaling the data, to compare taxa abundances, samples diversity, and functional profiles. This allowed to evaluate the influence of time, diet, and their combination on the microbiome composition and functionality. Heatmaps were also generated to visually compare the functional profiles of different groups. This was achieved for each functional level by selecting the 30 most variable functional groups with the highest abundance. In order to determine a characteristic signature for each diet effect, phyla including no less than 10% of the species that were significantly different in at least one comparison were considered (P-value < 0.05). Species with an average abundance in the 2 diet groups less than 0.025% were discarded. The signature was obtained as the set of selected species that were also important in explaining the beta diversities (i.e., species that were found to be important also by SIMPER, as previously defined). The signature analysis was repeated for each time point. All statistical analyses were performed in python 2.7 and R 3.3.0.

As for productive traits, 1-way ANOVA was used to analyze the data considering the treatment as main effect. When the effects were found to be significant, treatment means were separated using the Student–Newman–Keuls test (SAS Institute, 1988) and results were considered different if $P < 0.05$.

RESULTS

Species Diversity Among Chickens fed Different Diets

The species diversity observed within the microbial population in microbiota colonizing the cecum contents of broilers fed the different diets at 14 and 42 D, quantified by the Pielou alpha diversity, was similar in both tested groups (Supplemental Figure S1). The principal coordinate analysis plots (Figure 1), generated using the Bray–Curtis distance metric, confirmed that, despite the strong relationship between age of the birds and microbiota composition, the microbiota compositional diversity (measured by the beta diversity parameter) did not differentiate samples according to the diets at family level, at 14 and 42 D (Figures 1A and 1B), and neither at species level (Figures 1C and 1D).

Impact of Diet and Age of the Birds on Microbiota Composition

At phylum level, Firmicutes accounted for up to 70% of the differences observed between all pairwise comparisons (i.e., diet vs. time) at both 14 (Figure 2A) and 42 (Figure 2B) D. The additional phyla accounting for such differences were Actinobacteria and Proteobacteria, at both sampling times, as well as
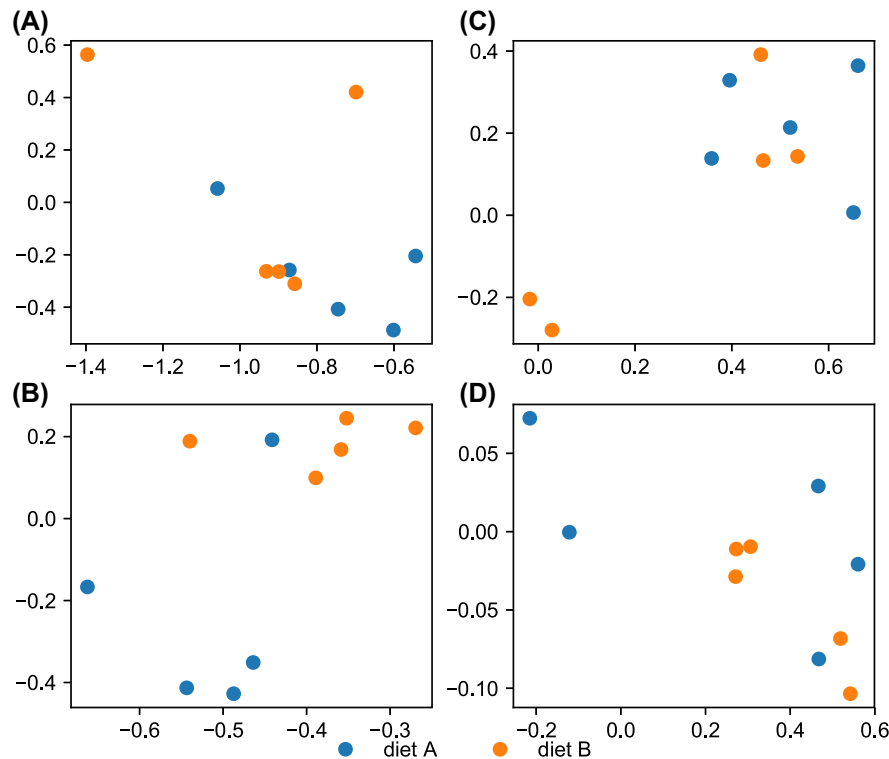
Figure 1. Principal coordinate analysis (PCoA) plots showing the impact of chickens' age and diets on microbiome composition in terms families and species. (A) Families 14 D; (B) families 42 D; (C) species 14 D; (D) species 42 F. The diets included in the comparison are the basal diet (diet A) and basal diet –7% crude protein (diet B).
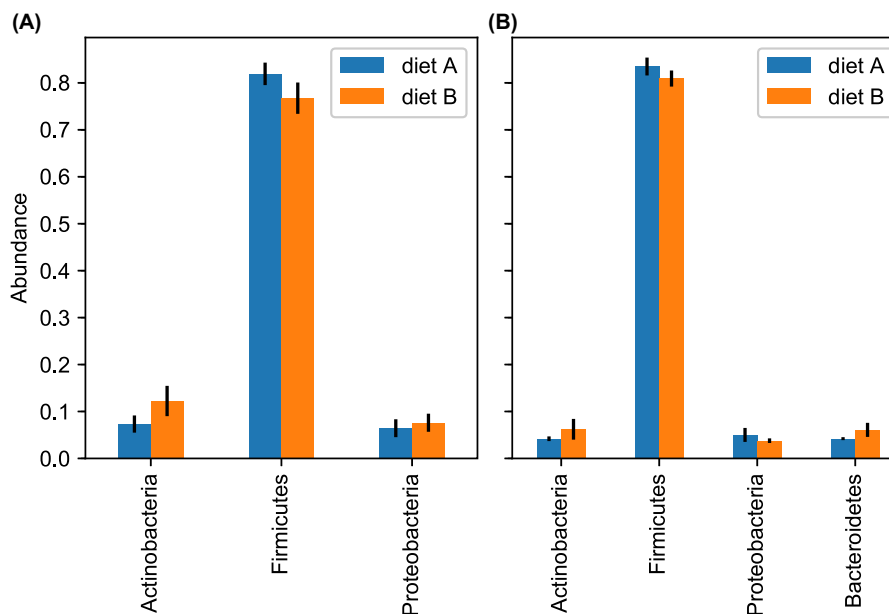

Figure 2. Phyla contributing to $\geq 70\%$ of the differences between microbiomes associated to chickens fed with the basal diet (diet A) and basal diet –7% crude protein (diet B). Such differences were calculated at 14 (A) and 42 (B) D using the Bray–Curtis distance.

Bacteroidetes at 42 D only (Figure 2B). At species level, *Faecalibacterium prausnitzii* was the most abundant species among those accounting for up to 70% of the differences observed at both 14 and 42 D within the phylum Firmicutes, followed by *Subdoligranulum variabile* at 14 D (Figure 3A) and *Pseudoflavonifractor*

capillosus at 42 D (Figure 3B). *Bifidobacterium longum* was the most abundant species among those accounting for up to 70% of the differences observed at both 14 (Figure 4A) and 42 (Figure 4B) D within the phylum Actinobacteria, followed by *Bifidobacterium adolescentis* at 14 D (Figure 4A). *Escherichia coli* was the most
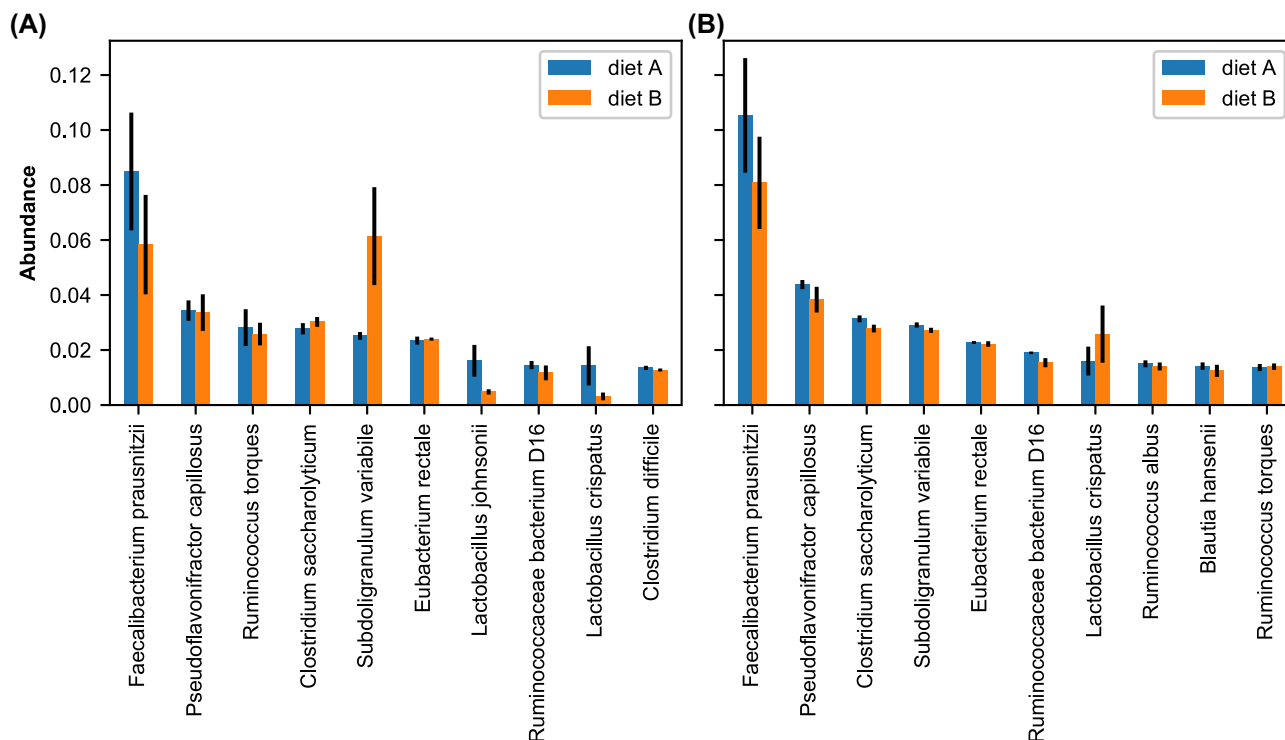
Figure 3. Firmicutes species contributing to $\geq 70\%$ of the differences between microbiomes associated to chickens fed with the basal diet (diet A) and basal diet –7% crude protein (diet B). Such differences were calculated at 14 (A) and 42 (B) D using the Bray–Curtis distance.

abundant species causing differences between pairwise comparisons at both sampling times within the phylum Proteobacteria, followed by *Salmonella enterica* and different species belonging to the genus *Shigella* at 14 D (Figure 5A) and by *Burkholderiales bacterium* 1_1_47, *Parasutterella excrementihominis*, and *Oxalobacter formigenes* at 42 D (Figure 5B). *Alistipes putredinis* accounted for the majority of the differences observed at species level between pairwise comparisons at both 14 (Figure 6A) and 42 (Figure 6B) D within the phylum Bacteroidetes, followed by *Alistipes shahii* and *Alistipes* sp. *HGB5* at 42 D (Figure 6B).

Overall, the abundance of *F. prausnitzii* in the ceca of chickens fed different diets did not significantly change at both 14 and 42 D and the same was observed for *E. coli* (Supplemental Figure S2). At both sampling times, the most abundant class was represented by Clostridia, followed by Bacilli, showing an abundance ≥ 0.592 in the ceca of both tested groups (Figure 7A). The class of Erysipelotrichia was stable at both sampling times in groups fed both diets (i.e., abundance ranging between 0.026 and 0.037), whereas Gammaproteobacteria decreased and Bacteroidia increased in both groups between 14 and 42 D (Figure 7A). At family level, the most abundant groups were Clostridiaceae and Ruminococcaceae (Figure 7B), followed by Lachnospiraceae, Eubacteriaceae, and Lactobacillaceae. Clostridiaceae and Eubacteriaceae showed abundances quite stable at both sampling times in the ceca of broilers fed both diets (i.e., abundances ranging between 0.183 and 0.192 for Clostridiaceae and

0.052 and 0.053 for Eubacteriaceae). Ruminococcaceae increased over time, between 0.186 and 0.214, in the ceca of broilers belonging to control group (A) and slightly decreased between 0.190 and 0.185 in low protein group (B). Lachnospiraceae slightly decreased over time in both group A (i.e., between 0.134 and 0.117) and group B (i.e., between 0.135 and 0.125). Finally, Lactobacillaceae slightly decreased over time in the ceca of broilers belonging to the control group (i.e., between 0.061 and 0.050) but then significantly increased in the low protein group and at 42 D were significantly higher than in the control group (i.e., 0.022 vs. 0.070) (Figure 7B).

Impact of Diet, Age and Their Interactions on Microbiota Composition

The ANOVA 2-way analysis, assessing the impact of diet, time and their interaction on abundance of species colonizing the ceca, showed that abundances of species belonging to the phyla Actinobacteria and Proteobacteria were mainly affected by the diet, as well as interaction between diet and time. On the contrary, abundances of species belonging to the phyla Firmicutes and Cyanobacteria were mainly affected by age of the birds (Figure 8). Overall, the number of species with significantly changed abundances in the ceca of broilers fed low protein diet in comparison to control diet was similar at both sampling times (i.e., 43 and 49 species at 14 and 42 D, respectively). At 14 D, the majority of the
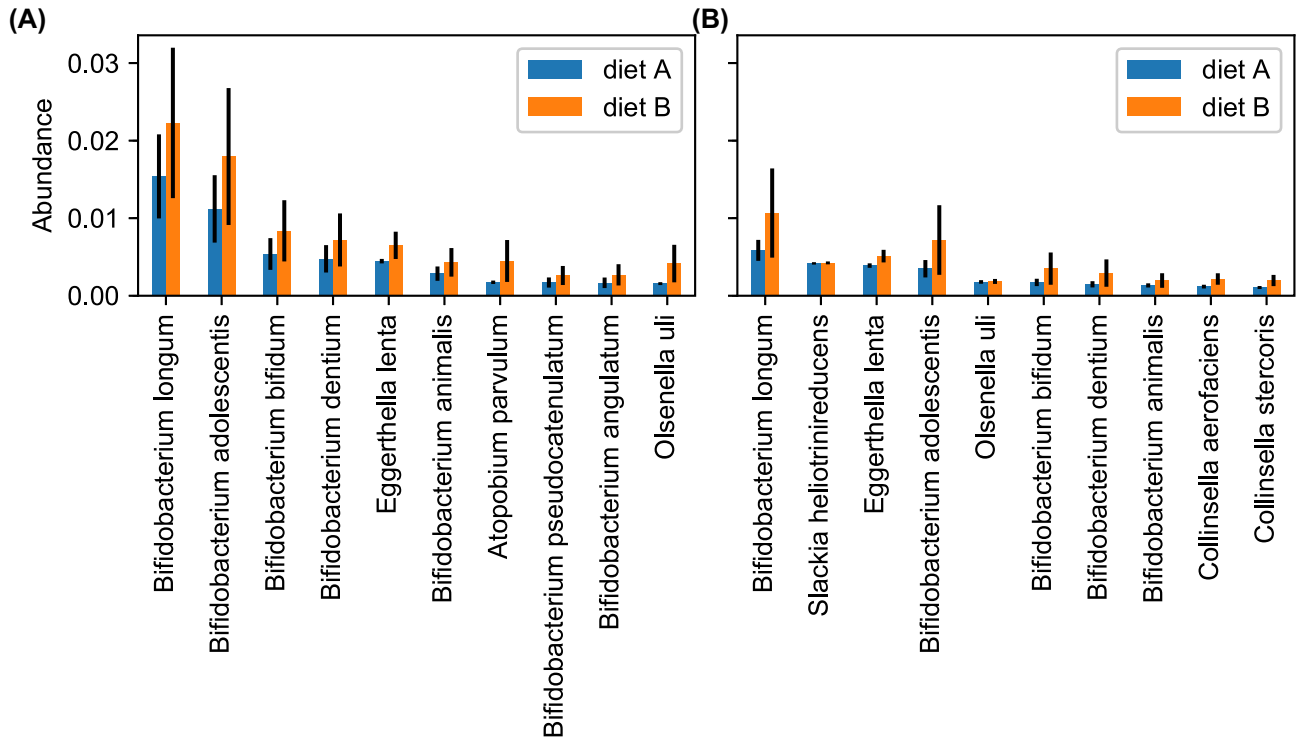
Figure 4. Bacteroidetes species contributing to ≥ 70% of the differences between microbiomes associated to chickens fed with the basal diet (diet A) and basal diet –7% crude protein (diet B). Such differences were calculated at 14 (A) and 42 (B) D using the Bray–Curtis distance.
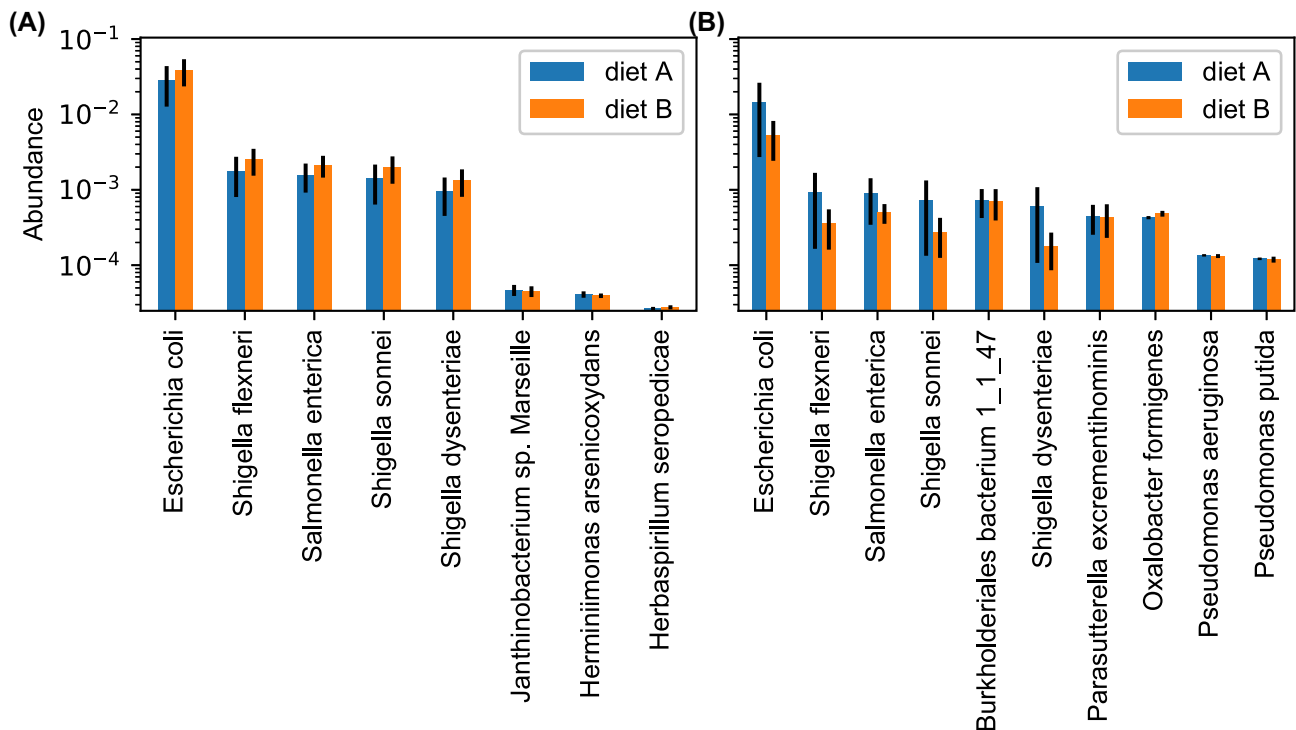

Figure 5. Proteobacteria species contributing to ≥ 70% of the differences between microbiomes associated to chickens fed with the basal diet (diet A) and basal diet –7% crude protein (diet B). Such differences were calculated at 14 (A) and 42 (B) D using the Bray–Curtis distance.

species with increased abundances in the ceca of broilers fed the low protein diet in comparison to the control diet belonged to the phyla Actinobacteria, whereas the species with decreased abundances belonged to the phyla Firmicutes and Proteobacteria (Figure 9A). At 42 D, most of the species with increased abundances in the ceca of broilers fed the low protein diet belonged to the phyla Firmicutes and those with decreased abundances to both Firmicutes and Proteobacteria (Figure 9B).
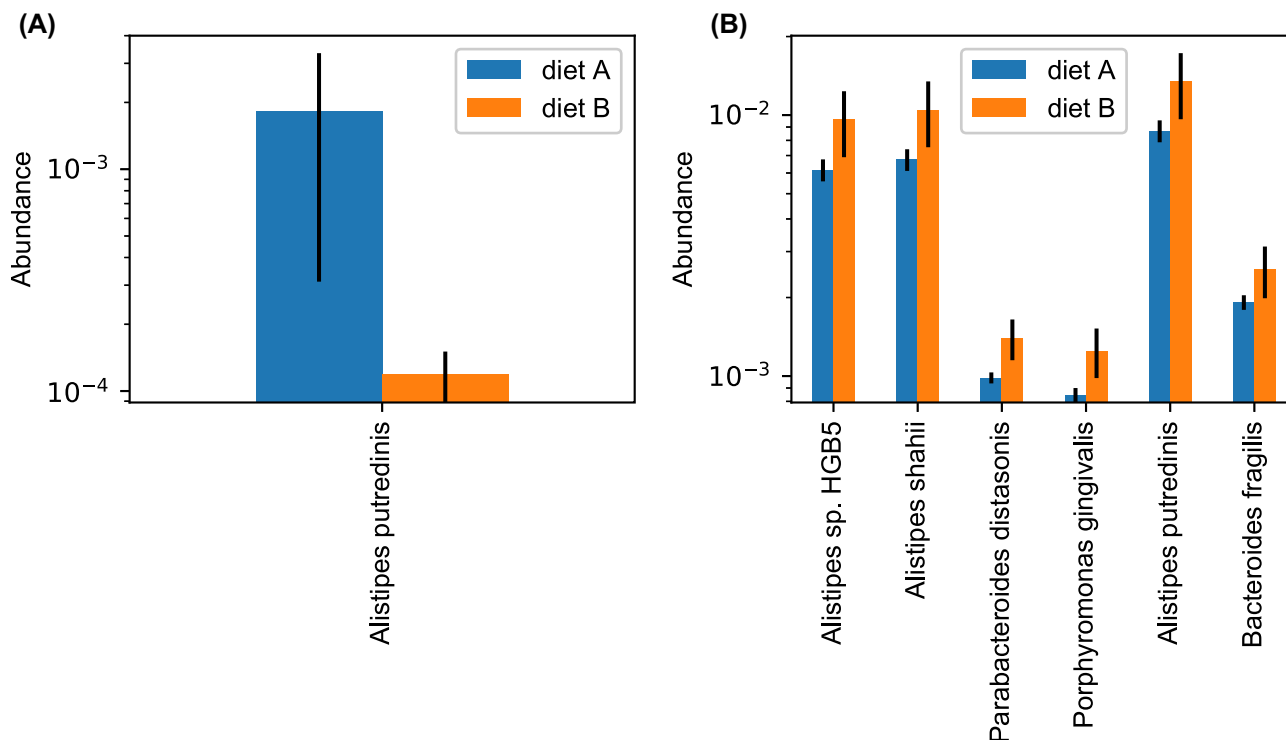
Figure 6. Actinobacteria species contributing to $\geq$ 70% of the differences between microbiomes associated to chickens fed with the basal diet (diet A) and basal diet –7% crude protein (diet B). Such differences were calculated at 14 (A) and 42 (B) D using the Bray–Curtis distance.

Identification of Signature Species

At each sampling time, the species showing in the t test a P value < 0.05 in comparison to the control (i.e., diet A) and an average abundance $\geq$ 0.025% were considered signature species. At 14 D, 5 signature species were identified in the ceca of broilers fed the diet containing –7% crude protein (Table 2). All those species belonged to the phylum Firmicutes; *Eubacterium cylindroides* was significantly higher in the ceca of broilers fed the low protein diet in comparison to the control, whereas *Erysipelotrichaceae bacterium 3 1 53*, *Lactobacillus salivarius*, *Lactobacillus fermentum*, and *Lactobacillus brevis* were significantly lower (Table 2). At the end of the rearing period, the diet with –7% crude protein showed a significantly lower abundance of 3 signature species, represented by *Bacillus licheniformis*, *Lactococcus lactis*, and *Lactobacillus ruminis* (Table 2).

Functional Diversity Among Chickens fed Different Diets

The average abundances of genes coding for level 1 metabolic and functional protein categories showed comparable abundance in all tested groups. The most abundant genes were those coding for carbohydrates metabolism, followed by genes for clustering-based subsystems (Supplemental Figure S3). The average abundance of the 30 most variable level 2 functions was similar between the 2 tested diet groups, with

dominance of genes coding for protein biosynthesis, followed by genes included in a miscellaneous SEED category, counting a diverse set of genes identified during investigation of plant–prokaryote interactions by a project at the Department of Energy (Supplemental Figure S4). The most abundant genes coding for level 3 functions were those for sugar utilization in Thermotogales, followed by genes for DNA replication and universal GTPases (Supplemental Figure S5). The most abundant functional genes were cystine desulfurase, followed by alpha-galactosidase and serine hydroxymethyltransferase (Supplemental Figure S6).

No gene coding for level 1 function showed a mean value of abundance significantly different between diet groups at both sampling times. Concerning genes coding for level 2, level 3, and level functions significative differences were calculated for genes with a mean level of abundance always lower than 0.01 (Table 3). The only exception was represented by the genes coding for purines (Table 3).

Productive Performances

Productive performances, either evaluated at the end of each feeding phase or for the entire experimental period, are reported in Table 4. At the end of the starter phase (11 D), chickens from control group showed a body weight (BW) similar to that of the low protein group (i.e., 276 and 271 g, respectively) and a better FCR (i.e., 1669 and 1.711, P = 0.09). A similar trend was observed at the end of the grower I phase
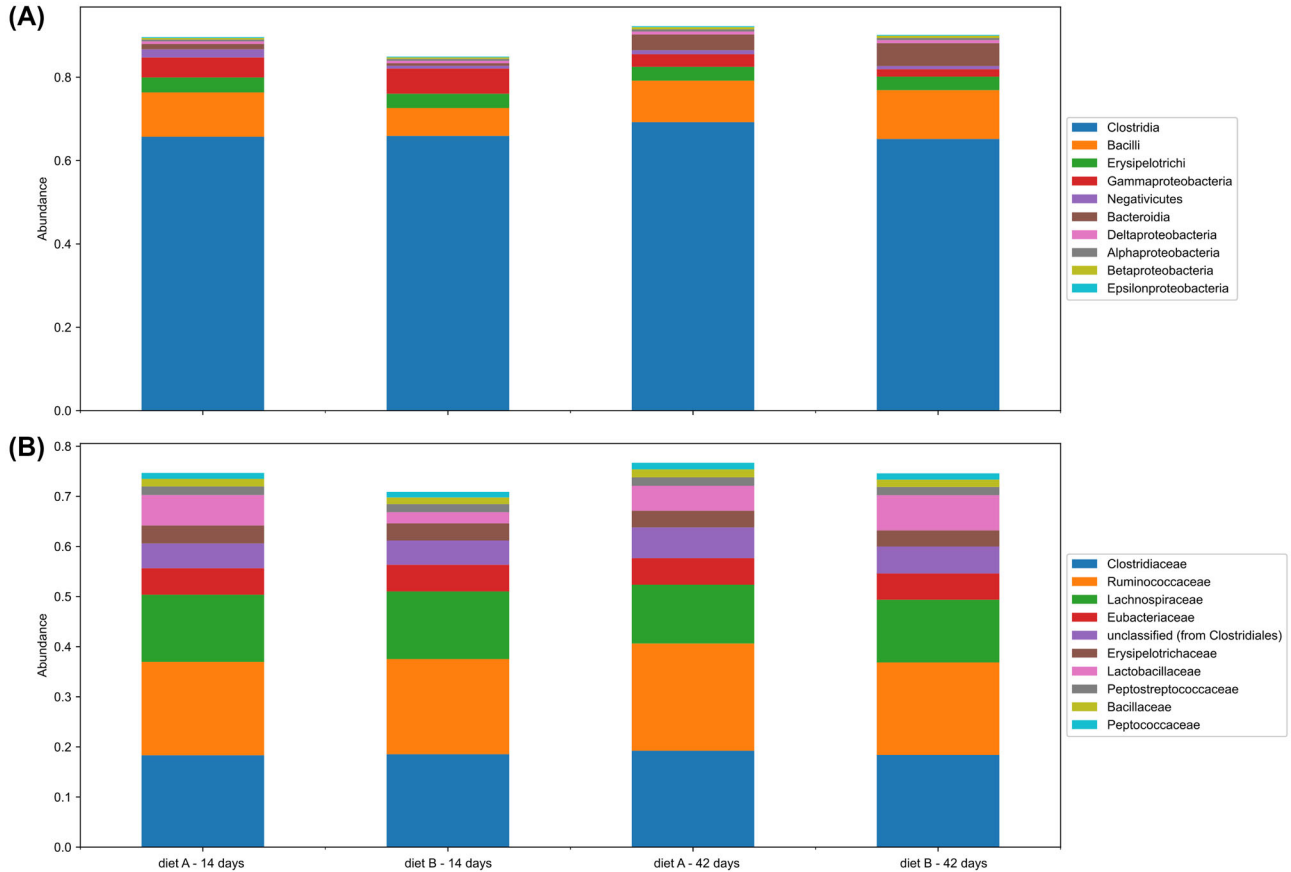
Figure 7. Abundances of bacteria belonging to the 10 most abundant phyla. (A) Class level; (B) family level. The x-axis indicates bacteria quantified in basal diet (diet A) at 14 D; basal diet –7% crude protein (diet B) at 14 D; basal diet (diet A) at 42 D; basal diet –7% crude protein (diet B) at 42 D.

(20 D) (i.e., BW 758 vs. 743 g and FCR 1.797 vs. 1.830 for control and low protein group, respectively) and at 35 D (i.e., BW 1.941 vs. 1.922 g and FCR 1.903 vs. 1.902 for control and low protein group, respectively). In the finisher phase, BWs resulted similar (2.722 vs. 2.716 g for control and low protein group, respectively), whereas FCR was significantly worse in the control group in comparison to the low protein group (i.e., 1.888 vs. 1.821, $P < 0.01$). DFI resulted similar among groups in all the feeding phases, with the exception of the finisher period, when chickens fed low protein diet showed a lower value in comparison to the control (i.e., 214.3 vs. 211.7 g/bird/d; $P = 0.08$). Overall, considering data of the entire experimental period (0 to 42 D), birds belonging to both diet groups reached a similar BW (i.e., 2.722 and 2.716 g for control and low protein group, respectively), DWG (63.1 vs. 63.0 g/bird/d), DFI (119.2 vs. 118.6 g/bird/d), and FCR (1.889 vs. 1.882). Concerning mortality, it resulted similar in both groups (Table 4). Figure 10 shows the crude protein intake calculated for each feeding phase and the entire rearing period. Overall, the crude protein intake was 1,036 and 954 g in chickens belonging to group A and B, respectively. This result confirms that chickens receiving the low crude protein diet did not adjust the feed intake to compensate the lower concentration of protein in their feed.
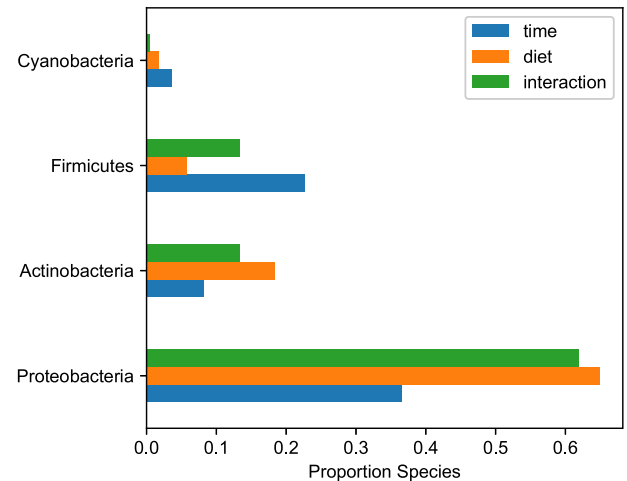

Figure 8. Phyla showing an abundance significantly changed according to sampling time, diet, or their interaction. The x-axis shows the proportion of significantly changed species belonging to the phyla shown in the y-axis.

DISCUSSION

In this study, the microbiomes colonizing the ceca of chickens fed a control diet and the same diet with a reduced level of crude protein (–7%) were investigated at 14 and 42 D to assess changes in both composition and relative frequency of abundance of microbial
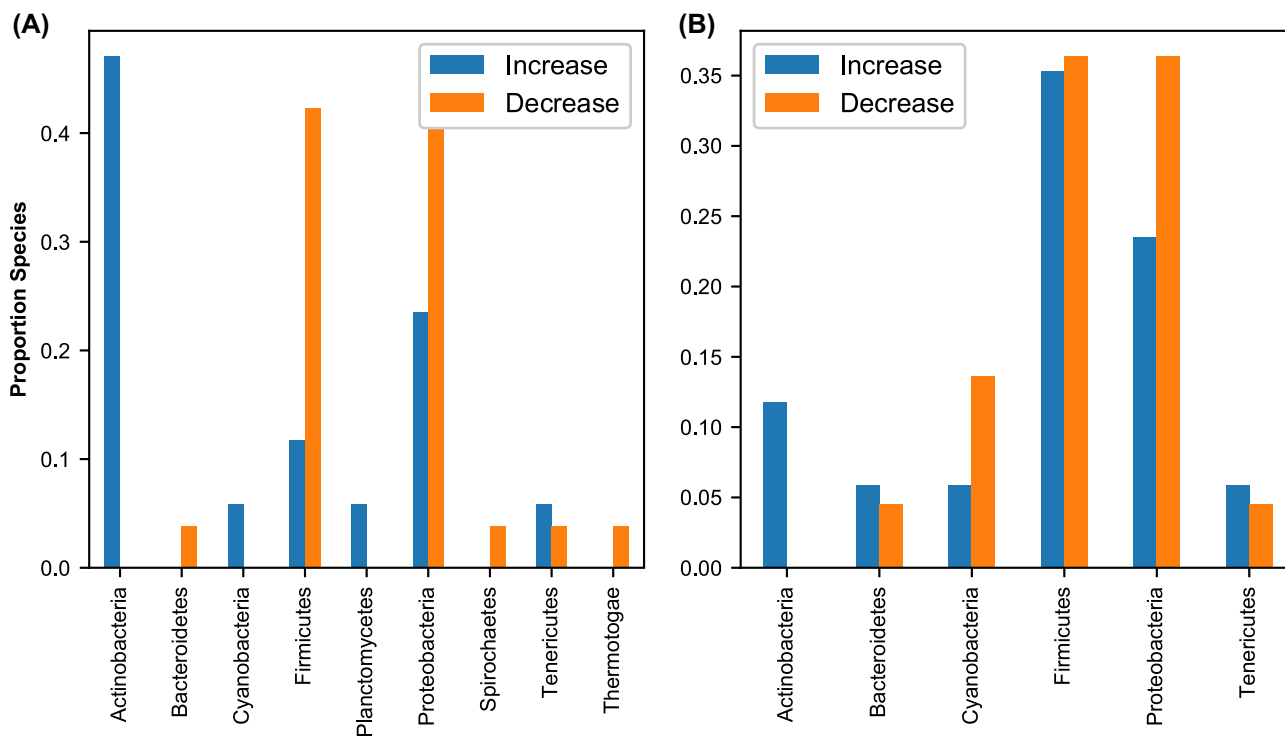
Figure 9. Proportion of species significantly increased or decreased in the ceca of broilers fed basal diet –7% crude protein (diet B) in comparison to the basal diet (diet A) at 14 (A) and 42 D (B).

species and functional genes. A strong relationship between age of the birds and microbial population colonizing the ceca was observed. Indeed, the microbiome compositional diversity did not group the samples at family and species level according to the diet, at both 14 and 42 D (Figure 1). However, the abundances of species belonging to the phyla Actinobacteria and Proteobacteria were mainly affected by the diet, as well as interaction between diet and time, whereas those of species belonging to the phyla Firmicutes and Cyanobacteria were mainly affected by age of the birds (Figure 8). These results prove that there are species whose abundance is affected by specific feed formulations, whereas others change only because of time. Regarding Cyanobacteria, detected at 14 D but not at 42 D, the results of our study confirm those of Xiao et al. (2017) who investigated the microbiota composition of 8 broilers at 42 D of age. In each broiler, 5 GITs were analyzed (i.e., duodenum, jejunum, ileum, cecum, and colon). The results showed that Cyanobacteria at 42 D were abundant in duodenum, jejunum, ileum, and colon, whereas nearly none was found in the cecum.

The lower level of protein in the diet decreased the abundance of Lactobacillaceae at 14 D but then they increased over time and at the end of the rearing period. Lactobacillaceae were significantly higher in the ceca of broilers fed low protein diet in comparison to the control group (Figure 7B). Lactobacillaceae have been considered key players in host metabolic balance because they may reduce the antigen load from gut

bacteria to the host and may alleviate certain inflammation responses (Zhu et al., 2015). The association between increase in Lactobacillaceae and improvement in FCR has been also observed by Yan et al., (2017) in hens. The authors suggested that *Lactobacillus* could generally improve the gut from pathogens and promote efficient nutrient and energy extraction in the host. In our study, the connection between Lactobacillaceae abundances and feed efficiency is confirmed at species level for *Lactobacillus crispatus* (Figure 3). At 14 D, this species was significantly lower in the low protein group in comparison to the control group. However, at the end of the rearing period (i.e., 42 D) it was significantly higher in the ceca of chickens fed the low protein diet showing also a better FCR (Figure 3 and Table 4). The link between *Lactobacillus* spp. in the ceca of broilers and FCR was also confirmed by Altaher et al. (2015) in chickens receiving a diet supplemented with *Lactobacillus pentosus* and *Lactobacillus acidipiscis*. On the contrary, Singh et al. (2015) investigated the difference in microbial communities between good and poor feed efficiency broilers using fecal samples and reported similar abundances of Lactobacillus and Bacteroides in the 2 groups. De Cesare et al. (2017) investigated the effects of the dietary supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) on cecum microbiome and productive performances in broiler chickens. These authors did not observed a significant increase in the mean relative frequency of abundance of Lactobacillaceae in the ceca of treated birds neither. However, they registered a better FCR in the treated

Table 2. Species assessed as signature, identified in the ceca of broilers fed basal diet –7% crude protein (group B) with *P* values < 0.05 in comparison to basal diet (group A) and with an average abundance ≥ 0.025% within the 3 most abundant phyla colonizing the birds at 14 and 42 D.

Phylum	Species	T-test *P*-value	SIMPER	Increase(+)/Decrease (−)
Group B 14 D				
Firmicutes	*Lactobacillus salivarius*	0.0368	0.5499	−
	Lactobacillus fermentum	0.0419	0.8324	−
	Eubacterium cylindroides	0.0451	0.8596	+
	Erysipelotrichaceae bacterium 3 1 53	0.0399	0.8998	−
	Lactobacillus brevis	0.0408	0.9033	−
Group B 42 D				
Firmicutes	*Bacillus licheniformis*	0.0471	0.9192	−
	Lactococcus lactis	0.0471	0.9141	−
	Lactobacillus ruminis	0.0385	0.9023	−

SIMPER—ordered cumulative contribution to the observed dissimilarity between the diet and the control.

Table 3. Pairwise t-test results on metabolic and functional protein categories at 14 and 42 D.

Function	Mean A	Mean B	*P*-value
Level 2–results with *P*-values <0.05 and abundance mean values ≥ 0.001			
14 D			
Protein secretion system, type VI	0.00103	0.00103	0.0286
42 D			
Purines	0.01472	0.01558	0.0386
CRISPs	0.00177	0.00145	0.0460
Level 3–results with *P*-values <0.05 and abundance mean values ≥ 0.0005			
14 D			
rRNA modification bacteria	0.00361	0.00335	0.0095
CBSS-262,719.3.peg.410	0.00134	0.00111	0.0271
Type VI secretion systems	0.00103	0.00125	0.0286
USS-DB-7	0.00099	0.00120	0.0477
42 D			
One-carbon metabolism by tetrahydropterines	0.00165	0.00185	0.0022
CBSS-306,254.1.peg.1508	0.00049	0.00056	0.0025
Sporulation Cluster	0.00148	0.00163	0.0074
Level functions—results with *P*-values <0.05 and abundance mean values ≥ 0.0002			
14 D			
PTS system, cellobiose-specific IIC component (EC 2.7.1.69)	0.00032	0.00017	0.0126
LSU ribosomal protein L11p (L12e)	0.00055	0.00045	0.0187
Dipeptide transport system permease protein DppC (TC 3.A.1.5.2)	0.00024	0.00032	0.0199
GTP-binding protein HflX	0.00043	0.00059	0.0203
UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2, 6-diaminopimelate-D-alanyl-D-alanine ligase (EC 6.3.2.10)	0.00021	0.00084	0.0338
N-acetyl-L, L-diaminopimelate aminotransferase (EC 2.6.1.-)	0.00021	0.00014	0.0349
Phosphoesterase, DHH family protein	0.00037	0.00026	0.0444
23S rRNA (Uracil-5-) -methyltransferase RumA (EC 2.1.1.-)	0.00073	0.00053	0.0453
42 D			
Site-specific recombinase, phage integrase family	0.00036	0.00027	0.0017

group in comparison to the control and interpreted this result as *L. acidophilus* supporting butyrate-producing species as *Ruminococcus obeum* and *Roseburia intestinalis* through a cross-feeding mechanism.

Concerning *F. prausnitzii,* Yan et al. (2017) reported that it was more abundant in poor feed efficiency hens. The same trend was observed in our study (Figure 3), but this does not reflect previous findings. According to Miquel et al. (2014), the increase of *B. longum* and *Lactobacilus* spp. might fed *F. prausnitzii* through the

production of acetate. In a study supporting this hypothesis, *B. longum* BB536 intake (13 wk treatment) enhanced *F. prausnitzii* 16S rRNA gene copy numbers in Japanese individuals with cedar pollinosis (Odamaki et al., 2007). Furthermore, an in vitro experiment conducted to quantify butyric-producing bacteria in a simulated broiler cecum, the supplementation of different *Lactobacillus* species, including *L. salivarius*, after 24 h of incubation, significantly increased the number of Lactobacilli, Bifidobacteria, and *F. prausnitzii*

Table 4. Productive performances of chickens fed basal diet (group A) and basal diet −7% crude protein (group B) separated for feeding phase and for overall rearing period.

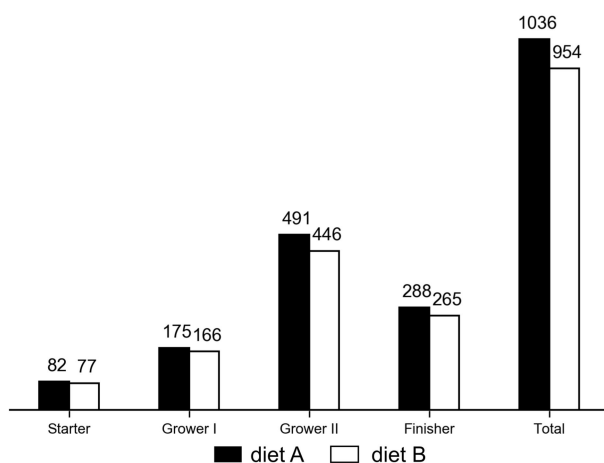|	Group A	Group B	SE	P-value
n.	g	g		
0 to 11 D				
Chick body weight (g)	40.6	40.9	0.14	0.08
Body weight (g)	275.7	271.2	3.69	0.23
Daily weight gain (g/bird/d)*	19.6	19.2	0.31	0.21
Daily feed intake (g/bird/d)*	32.7	32.8	0.41	0.72
Feed conversion rate*	1.669	1.711	0.02	0.09
Mortality (%)	0.15	0.00	0.04	0.33
12 to 20 D				
Body weight (g/bird)	758	743	16.8	0.39
Daily weight gain (g/bird/d)*	53.1	51.9	1.55	0.44
Daily feed intake (g/bird/d)*	95.2	94.8	2.15	0.88
Feed conversion rate*	1.797	1.830	0.03	0.36
Mortality (%)	0.34	0.68	0.03	0.35
21 to 35 D				
Body weight (g/bird)	1,941	1,922	21.8	0.38
Daily weight gain (g/bird/d)*	86.8	86.7	0.72	0.94
Daily feed intake (g/bird/d)*	165.2	165.0	1.12	0.88
Feed conversion rate*	1.903	1.902	0.01	0.95
Mortality (%)	0.34	0.34	0.03	1.00
36 to 42 D				
Body weight (g/bird)	2,722	2716	25.9	0.82
Daily weight gain (g/bird/d)*	113.5	116.3	1.41	0.07
Daily feed intake (g/bird/d)*	214.3	211.7	1.39	0.08
Feed conversion rate*	1.888 B	1.821 A	0.02	<0.01
Mortality (%)	0.17	0.00	0.01	0.33
0–42 d				
Body weight (g/bird)	2,722	2,716	25.9	0.82
Daily weight gain (g/bird/d)*	63.1	63.0	0.59	0.91
Daily feed intake (g/bird/d)*	119.2	118.6	0.87	0.54
Feed conversion rate*	1.889	1.882	0.01	0.50
Mortality (%)	1.00	1.02	0.03	1.00

*Corrected for mortality.
A, B: $P < 0.01$.

Figure 10. Crude protein intake of chickens from basal diet (diet A) and basal diet −7% crude protein (diet B) during each feeding phase and the entire rearing period.

(Meimandipour et al., 2010). *F. praustnizii* and *S. variabile* are producers of short-chain fatty acids, such as butyric acid and formic acid (Bjerrum et al., 2006), having an important function in both growth performance (Garcia et al., 2007) and protection against pathogens (Fernadez-Rubio et al., 2009). In our study, *S. variabile* was significantly higher in the ceca of broilers fed the low protein diet at 14 D when birds showed a poor feed

efficiency. However, this difference between abundance of *S. variabile* in the ceca of broilers fed the low protein diet and the control diet was lost on time (Figure 3).

The most abundant functional genes in all tested groups were cystine desulfurase, followed by alpha-galactosidase and serine hydroxymethyltransferase. Cysteine desulfurase is a pyridoxal 5′-phosphate-dependent homodimeric enzyme that catalyzes the conversion of L-cysteine to L-alanine and sulfane sulfur via the formation of a protein-bound cysteine persulfide intermediate on a conserved cysteine residue. Increased evidence for the functions of cysteine desulfurases has revealed their important roles in the biosyntheses of Fe-S clusters, thiamine, thionucleosides in tRNA, biotin, lipoic acid, molybdopterin, and NAD. These enzymes are also proposed to be involved in cellular iron homeostasis and in the biosynthesis of selenoproteins (Mihara and Esaki, 2002). At 14 D, genes coding protein secretion system type VI (T6SS) were significantly higher in the ceca of birds fed −7% crude protein in comparison to control group (Table 3). However, this difference was lost on time and at 42 D the same genes showed comparable levels in both groups. Type VI secretion systems (T6SSs) are the most recently described specialized secretion systems. T6SSs are widely distributed in Gram-negative bacteria, especially in Proteobacteria, where type VI secretion gene clusters may be found in several copies on the chromosome (Cascales and Cambillau 2012). First thought of as secretion systems dedicated to virulence towards eukaryotic host cells, recent data have shown unambiguously that these systems are regulating bacterial interactions and competition (Cascales and Cambillau 2012). T6SSs are required to kill neighboring, non-immune bacterial cells by secreting antibacterial proteins directly into the periplasm of the target cells upon cell-to-cell contact. This intense bacterial warfare indirectly contributes to pathogenesis in animals as T6SS facilitates the colonization of specific niches where pathogens then develop anti-host defenses and toxins (Cascales and Cambillau 2012).

In relation to the productive performances measured in this study, the FCR was better in the control group in comparison to the low protein group between 0 and 11 D as well as 12 and 20 D (Table 4). Afterwards, between 21 and 35 D, the same productive parameter was similar in the 2 diet groups but in the last rearing period, ranging between 36 and 42 D, it was significantly better in the low protein group in comparison to the control. Since the level of crude protein was the only factor different between the 2 diet groups, it seems appropriate to link the significant differences in FCR to the reduction of crude protein in the diet. Indeed, in our trial the crude protein intake was 1,036 and 954 g in chickens belonging to control group and low protein group, respectively (Figure 10), showing that chickens receiving the low crude protein diet did not adjust the feed intake to compensate the lower concentration of protein in their feed.

In conclusion, this study showed, for the first time, that a reduced intake of crude protein (i.e., 82 g/bird for the entire rearing period) in broilers increases the abundance of Lactobacillaceae in the ceca and this could be linked to a better FCR between 36 and 42 D. A reduced intake of crude protein in broiler chicken production can help to improve the exploitation of edible resources, while reducing the emission of nitrogen pollutants in the environment.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Supplemental Figure S1. Pielou-alpha diversity calculated for the species colonizing the ceca of broilers fed basal diet (group A) and basal diet –7% crude protein (group B) at 14 (A) and 42 D (B).

Supplemental Figure S2. Boxplots showing the distribution of *Faecalibacterium prausnitzii* at 14 (A) and 42 (B) D and the distribution of *Escherichia coli* at 14 (C) and 42 (D) D.

Supplemental Figure S3. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (level 1). The columns represent the diets at each sampling time, the rows represent the SEED subsystems (level 1), and the color bars represent the range of average relative abundance of reads annotated to each category.

Supplemental Figure S4. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (level 2). The columns represent the diets at each sampling time, the rows represent the SEED subsystems (level 2), and the color bars represent the range of average relative abundance of reads annotated to each category.

Supplemental Figure S5. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (level 3). The columns represent the diets at each sampling time, the rows represent the SEED subsystems (level 3), and the color bars represent the range of average relative abundance of reads annotated to each category.

Supplemental Figure S6. Heatmap showing the average relative abundance of metagenomic reads annotated to SEED Subsystems (function). The columns represent the diet types at each sampling time, the rows represent the SEED subsystems (function), and the color bars represent the range of average relative abundance of reads annotated to each category.

ACKNOWLEDGMENTS

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