

RESEARCH ARTICLE

Green forage and fattening duration differentially modulate cecal microbiome of Wanxi white geese

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Abstract

Gut microbial ecology is responsible for fatty acid metabolism in ruminants. The cecal microbiota composition of geese and their adaptation to fiber inclusion and feeding times were investigated in this study. A total of 116 Wanxi white geese were randomly selected at 70 days old. Eight geese were subjected to cecal sampling at 70 d of age, and the remaining 108 geese were divided into four groups with three replicates each (9 geese in each replicate). The geese in the four groups were fed 0, 15, 30, and 45% green forage (relative to dry matter), respectively. Three birds from each replicate were selected for cecal sampling at 80, 90, and 100 days old. All samples were subjected to 16S rRNA gene sequencing using the Illumina Ion Personal Genome Machine platform. Bacterial abundance was analyzed using two-way ANOVA analysis, and the relationship between the relative abundance of bacteria (phylum level) and fatty acids was analyzed using canonical correspondence analysis. Cecal microbiota in geese were mainly composed of *Bacteroidetes* (68.46%), *Firmicutes* (20.04%), and *Proteobacteria* (7.89%). Dietary treatments had no significant effect on the α -diversity indices of the cecal bacterial community ($P > 0.05$), but a numerical increase occurred with increased fattening duration and green forage inclusion. The *Selenomonadales* order ($P = 0.024$), *Negativicutes* class ($P = 0.026$), and *Megamonas* ($P = 0.012$) and *Oscillospira* ($P = 0.042$) genera were affected by green forage inclusion level, and microflora abundance was mainly influenced by the fattening duration. Bacteria phyla were mostly set along the line of linolenic acid and oleic acid. Finally, *Bacteroidales* might be an intestinal promoter that improves unsaturated fatty acid synthesis in geese.

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1. Introduction

Dietary fiber is a major component of plant cell wall, and it consists of both non-starch polysaccharides and non-carbohydrate component. Previous experiments reported that the fiber source in poultry diets plays an important role in gastrointestinal development and, ultimately,

energy metabolism [1]. Inefficient and variable responses to dietary fiber result from the absence of digestive enzymes for fiber and the relatively short digestive tract and digestive transition time associated with poultry gastrointestinal tracts [2,3]. Products from ruminants fed high fiber diet display increased bioactive unsaturated fatty acid (UFA) content [4]. Geese are herbivorous poultry that possess a relatively highly developed cecum with hemicellulose and cellulose digestibility of 41.5% and 17.4%, respectively, aided by the actions of cecal microbiota [5]. The complex composition of cecal microbiota can digest dietary fiber into lower fatty acids (FAs) to make it more dissolvable and absorbable. Thus, the addition of dietary fiber to goose feed could not only increase digestive transit time, but also promote nutrient absorption [6]. Traditionally, geese bred for meat are mainly reared outside with free access to grass. In modern husbandry, intensified geese production has led to the use of concentrated diets similar to those of broilers and ducks, which caused rapid growth and fat deposition in the abdomen with reduced UFA content [7]. Thus, there is an urgent need to formulate proper green forage as an additional dietary supplement during the geese fattening process.

Research has determined that gut microbial ecology is responsible for FA metabolism in ruminants [8], and several studies of cecal microbiota characteristics of broilers and laying hens were recently reported [3, 9–11]. However, little is known about the composition of goose cecal microbiota and their adaptation to fiber addition and feeding time. In addition, research is needed to clarify whether the gut bacteria in geese are also related to FA synthesis in a manner that is similar to that observed in ruminants. Wanxi white geese, a globally known Chinese indigenous breed, usually undergo fattening between 70 and 100 days old. Therefore, the present study aims to characterize the composition of microbiota in the cecum of Wanxi white geese during fattening with or without inclusion of different ratios of green forage, and to determine the relationship between microbiota and breast FAs composition.

2. Materials and methods

All experimental procedures were performed in accordance with guidelines developed by the China Council on Animal Care and Protocols and were approved by the Animal Care and Use Committee of Anhui Agricultural University, China.

2.1. Grouping and treatment of geese

A total of 116 male Wanxi white geese, obtained from Anhui Wanxi White Goose Preservation Farm, were raised in a floor system in open-sided houses. All geese were fed a commercial maize-soybean-based diet containing 155 g crude protein/kg feed with a metabolizable energy content of 15.11 to 15.89 MJ/kg feed. At 70 days old, 8 geese were randomly selected, weighed and then for sampling. The left 108 geese were individually weighed, and were randomly allocated to one of the four following green forage treatment groups (three replicates with nine geese per replication), and were treated as indicated: Group 1 was fed a concentrated diet without green forage supplements (white cabbage); Groups 2, 3, and 4 were fed 15, 30, and 45% green forage (relative to dry matter) plus a concentrated diet, respectively (Table 1). Geese were first fed green forage in each day from 8:00 am. Concentrated diet was then provided after green forage been consumed completely. Concentrated diet consumption was recorded in each replicate.

2.2. Sample collection and genomic DNA extraction

On day 70, eight geese were randomly selected before being grouped, and were then euthanized for gut sampling after a 12 h fast. Three geese from each replicate were randomly selected at the age of 80, 90, and 100 d, and were then euthanized for gut sampling after fasting for 12

Table 1. Nutrient composition of the experimental diets for Wanxi white geese from 70 d to 100 d of age.

| chemical composition | percentage of green forage relative to dry matter | | | |
|-----------------------------|---|-------|-------|-------|
| | 0 | 15 | 30 | 45 |
| water content % | 11.14 | 12.65 | 13.78 | 14.12 |
| ash % | 7.11 | 7.02 | 6.95 | 6.82 |
| crude protein % | 16.11 | 15.94 | 15.72 | 15.37 |
| crude fat % | 1.98 | 1.92 | 2.00 | 1.96 |
| crude fibre % | 3.19 | 5.34 | 5.69 | 5.88 |
| calcium % | 2.00 | 1.98 | 1.96 | 1.92 |
| potassium % | 0.60 | 0.67 | 0.72 | 0.77 |
| metabolisable energy, MJ/kg | 15.89 | 15.42 | 15.21 | 15.11 |

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h. Cecal samples were quickly collected and stored at -80 °C before DNA extraction. Goose was first administered a 10 ml 53 °C alcohol directly into the crop via oral gavage. About 30 minutes later after gavage, goose was euthanized by carbon dioxide inhalation and was bled immediately after euthanasia. After the body temperature was completely cooled down, breast muscle (*pectoralis major*) was collected and immediately stored in -20 °C for fatty acid composition analysis.

Total genomic DNA was extracted from 150–200 mg of frozen cecal contents using a QIAamp DNA Stool Mini Kit (QIAGEN, USA). The quality of the extracted genomic DNA was determined using a Qubit 2.0 fluorometer (Invitrogen, Q32866, California, USA). Agarose gel electrophoresis of genomic DNA showed a band with a molecular weight > 10kb and no degradation.

2.3. Microbial sequencing

The V3-V4 region of 16S rDNA was amplified using a pair of universal primers (334F: CCTACGGGAGGCAGCAG and 519R: ATTACCGCGGCTGCTGG). The 50 µL PCR amplification reaction volume consisted of 2 µL Taq enzyme, 1 µL 10 µM primers, 5 µL genomic DNA (30 ng), and water up to 50 µL. The PCR protocol was as follows: initial denaturation at 98 °C for 45 s; eight cycles at 98 °C for 15 s, 57 °C for 30 s, and 72 °C for 60 s; and a final elongation step at 72 °C for 60 s. The amplified PCR products were purified using a Magnetic Stand-96 (Life Technologies, AM10027). Products were then quantified using a Qubit 2.0 fluorometer (Invitrogen, Q32866), and the lengths of the amplified fragments were detected using an Agilent 2100 Bioanalyzer (Agilent, G2939AA). The amplified products were purified using magnetic beads. The concentration was determined using a Qubit2.0, and fragment distribution was detected using an Agilent 2100 Bioanalyzer. The quantified PCR fragments were used to construct libraries using an Ion Plus Fragment Library kit (Life Technologies, USA) according to the manufacturer's instructions. Library sequencing was performed using an Ion Personal Genome Machine (PGM) system (Life Technology, USA) with an Ion™316 Chip and an Ion PGM Sequencing 200 Kit v2 (Life Technology, 4482006), according to the manufacturer's guidelines.

2.4. Fatty acid content and composition analysis

The fatty acid profile was determined after the homogenized fat samples (or dried green cabbage) were defrosted. A 2 g sample was extracted by using chloroform:methanol (2:1, v/v) solution according to the Folch's method and esterified with methyl alcohol (93%) containing HCL (3%), then applied to DEGS column (DB-WAX, 0.25mm× 0.25µ,) and were detected

with FID by gas chromatography (Agilent GC7890A). Proportions of fatty acids are reported as percentages of total fatty acids by mass.

2.5. Data analysis

The sequenced Ion PGM raw reads were quality filtered using the FastX Toolkit 0.0.13. Valid reads were then clustered into operational taxonomical units (OTUs) using the Ribosomal Database Project (RDP) classifier method, OTUs were then aligned using the Silva database (<http://www.arb-silva.de>). OTUs that exhibited at least 97% nucleotide similarity were used for alpha diversity (Shannon and Simpson), richness (ACE and Chao1), Good's coverage, Venn diagrams, and rarefaction curve analyses using Mothur software (version 1.34.0, <http://www.mothur.org>). OTUs were taxonomically categorized using the naïve Bayesian RDP classifier found in the Silva database, with a minimum confidence score of 0.8. For downstream analysis, OTUs were filtered by discarding those that comprised fewer than 0.03 of all sequences. To evaluate the α -diversity of the samples, the rarefaction curves of phylogenetic diversity (PD) and a number of observed OTUs were computed using Mothur software. To normalize sequencing depth, the lowest counts among the samples were randomly subsampled in each library 1,000 times, and average values were used to measure diversity indices. The differences between mean values were identified using an analysis of two-way anova test conducted with SAS 9.3 software (SAS Inst. Inc., Cary, NC, USA). Microbial in each taxonomy level that were significantly different among treatment groups were further regressed on green forage supplementation using the following equation:

$$y = a + bx$$

Data for fatty acid (FA) content was analyzed by using PROC MIXED of SAS. The statistical model used included green forage, fattening duration and green forage \times fattening duration as fixed effects, and geese as random effect. A canonical correspondence analysis (CCA) was used to analyze the relationship between the relative abundance of bacteria (at the phylum level) and FA contents [12].

3. Results

3.1. Data summary

A total of 3,724,695 reads from the ceca microbial community were identified after filtering the sequences for quality control, sequencing errors, and chimeras. A total of 2,426,622 valid reads were screened by comparing the paired-end primers with a valid proportion of 63.74%. The total number of reads and valid reads from the original FASTQ files of each sample are presented in [S1 Table](#). There were 9,286 OTUs obtained using the RDP classifier with a 97% confidence rating when compared to the Geese gut microbial Database. Each OTU was then classified based on kingdom, phylum, class, order, family, genus, and species levels with 9,286, 3,952, 3,633, 3,633, 2,604, 1,580, and 10 OTUs in each level, respectively. The most abundant OTUs in each level are presented in [Table 2](#) and [Fig 1](#).

3.2. Effects of fattening duration on cecal microbiota

To assess the community α -diversity, the number of observed OTUs (at the 97% level) and PD values were calculated to evaluate whether the sequencing level was adequate for analysis. Rarefaction curves for the observed OTUs, Shannon-Wiener curves, and rank abundance curves approached a plateau, indicating that the sequencing depth was sufficient for the coverage of all OTUs present in the cecal samples ([Fig 2](#)). None of the dietary treatments had significant

Table 2. The top abundant operational taxonomical units (OTUs) at each level.

| Level | Top OUT category description | OUT Numbers |
|------------|---------------------------------------|-------------|
| (Taxonomy) | (Bacteria) | 9,286 |
| phylum | <i>Firmicutes</i> | 3,952 |
| class | <i>Bacteroidia</i> | 3,633 |
| order | <i>Bacteroidales</i> | 3,633 |
| family | <i>Ruminococcaceae</i> | 2,604 |
| genus | <i>Bacteroides</i> | 1,580 |
| species | <i>Bacteroides helcogenes</i> P36-108 | 10 |

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effects on the α -diversity indices of the cecal bacterial community ($P > 0.05$, Fig 2A–2F). However, α -diversity exhibited a numerical, but not significant, increase with increasing fattening durations. PD values also differed with fattening duration, but the differences were not statistically significant (Fig 3A and 3B). Rarefaction curves for the observed OTUs and PD values approached a plateau, indicating that the sequencing depth was sufficient for the coverage of all OTUs present in the cecal samples.

3.3. Effects of green forage supplementation on cecal microbiota

Rarefaction curves of 8,000 subsampled reads in the cecum showed comparable numbers of OTUs (at the 97% identity level) for each green forage supplemented group (Fig 4A and 4B), and there were no apparent differences in the rarefaction curves. However, numerical differences were found with regard to the lowest OTUs in cecal samples from geese fed a diet without green forage supplementation as well as the highest OTUs in those from geese fed diets with 30–45% green forage supplementation. Rarefaction for the PD values also exhibited the same trend with the OTU values. Rarefaction curves approached the saturation plateau at a sequencing depth of 2,000 in all sequenced samples.

3.4. Effects of fattening duration and green forage treatments on the α -diversity of cecal microbiota

Comparisons of the effects of fattening duration and green forage inclusion levels (Fig 5) indicated that the cecal microbial community of geese fed without green forage was significantly different from those of geese fed 15, 30, and 45% green forage ($P = 0.001$, $P < 0.001$, and $P < 0.001$, respectively). Furthermore, before 90 days of age, the cecal microbial community of those fed 15% green forage differed from those fed 30% green forage ($P < 0.001$). No significant differences were observed between geese fed 30% and 45% green forage ($P = 0.598$).

3.5. Microbial community structure at each taxonomic level

OTUs were taxonomically classified into different levels using the RDP classifier at a 97% confidence threshold. The classification of sequences from samples resulted in 22 phyla, 47 classes, 79 orders, 147 families, and 300 genera (S2 Table and S1–S5 Figs). The relative abundance of OTUs occupying $> 1\%$ was used for analyses at different levels (i.e., from phyla to genera) and compared among groups.

At the phylum level (Table 3, S6A Fig), the cecal microbiota community was mainly composed of *Bacteroidetes* (68.46%), *Firmicutes* (20.04%), and *Proteobacteria* (7.89%). No significant difference was detected in the microbiota composition at the phylum level among the green forage-fed groups. However, the percentage of *Bacteroidetes* and *Spirochaetae* in cecal

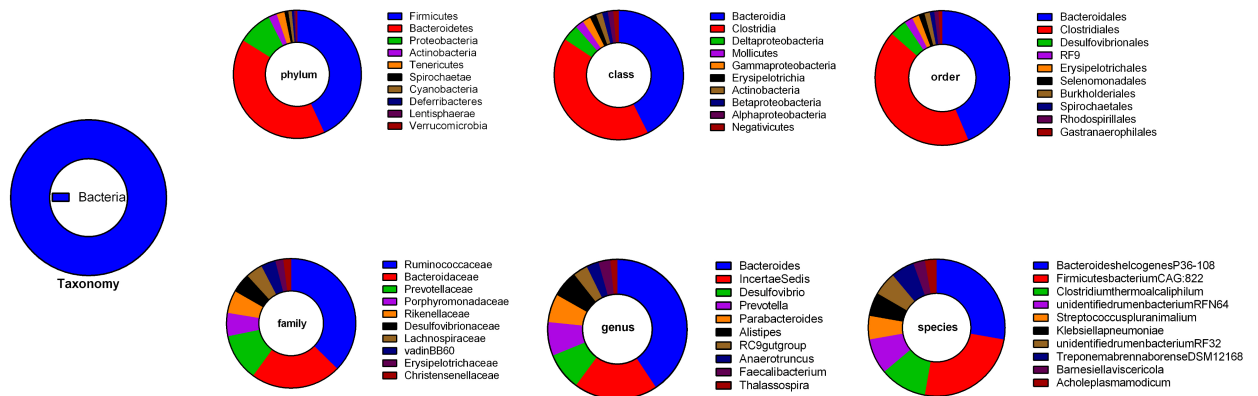


Fig 1. Ten most abundant operational taxonomical units (OTUs) at each level.

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samples of geese differed based on the fattening time ($P = 0.041$). The percentage of *Bacteroidetes* in cecal samples from geese that were fattened for 10 days was significantly higher than that of geese fattened for 20 or 30 days ($P = 0.023$ and $P = 0.047$, respectively). The percentage of *Spirochaetae* in cecal samples of geese before fattening was significantly lower than that of geese fattened for 10 and 20 days ($P = 0.043$ and $P = 0.001$, respectively).

At the order level (Table 4 and S6B Fig), *Clostridiales* was the second most abundant, and it exhibited a time-dependent increase ($P = 0.004$, $R^2 = 0.991$). *Clostridiales* constituted 9.02 and 17.70% before and 30 days after fattening, respectively. *Bacteroidales* exhibited a significantly different abundance based on the different fattening durations ($P = 0.021$). The *Selenomonadales* percentage exhibited a strong negative linear relationship with the fattening duration ($P = 0.023$, $R^2 = 0.955$), with the highest percentage occurring in geese before fattening ($P < 0.0001$). The percentage of *Spirochaetales* in the cecal samples of geese before fattening was significantly lower than that of geese fattened for 10, 20, and 30 days ($P = 0.001$), but no significant regression with fattening duration was observed ($P = 0.400$, $R^2 = 0.359$).

The percentage of *Selenomonadales* exhibited a strong negative linear relationship with green forage supplementation ($P = 0.029$, $R^2 = 0.942$), indicating that longer fattening durations resulted in lower *Selenomonadales* percentages in the cecal samples of geese fed a diet without green forage supplementation than in samples of those fed diets supplemented with 30 or 45% green forage ($P = 0.024$).

At the class level (Table 5 and S6C Fig), the most abundant microbial community was *Bacteroidia*, followed by *Clostridia*, *Deltaproteobacteria*, and *Negativicutes*. The *Negativicutes* percentage exhibited a strong negative linear relationship with fattening time ($P = 0.023$, $R^2 = 0.954$), indicating that a longer fattening time resulted in a lower percentage of *Negativicutes*. However, the highest *Negativicutes* percentage was found in the cecal samples of geese fed a diet without green forage supplementation ($P < 0.0001$). The *Clostridia* percentage exhibited a strong positive linear relationship with fattening time ($P = 0.004$, $R^2 = 0.992$), indicating that a longer fattening time resulted in a higher percentage of *Clostridia*, and the highest percentage was detected in the cecal samples of geese fed a diet with 45% green forage supplementation ($P < 0.0001$).

No significant linear relationship was detected between the percentage of *Spirochaetes* and *Bacteroidia* and fattening time in geese. However, the percentage of *Spirochaetes* from geese cecal samples collected before fattening was significantly lower than that of geese fattened for 10 and 20 days ($P = 0.044$ and $P = 0.001$, respectively). Furthermore, the percentage of *Bacteroidia* was higher in the cecal samples of geese before fattening and those fattened for 10 days

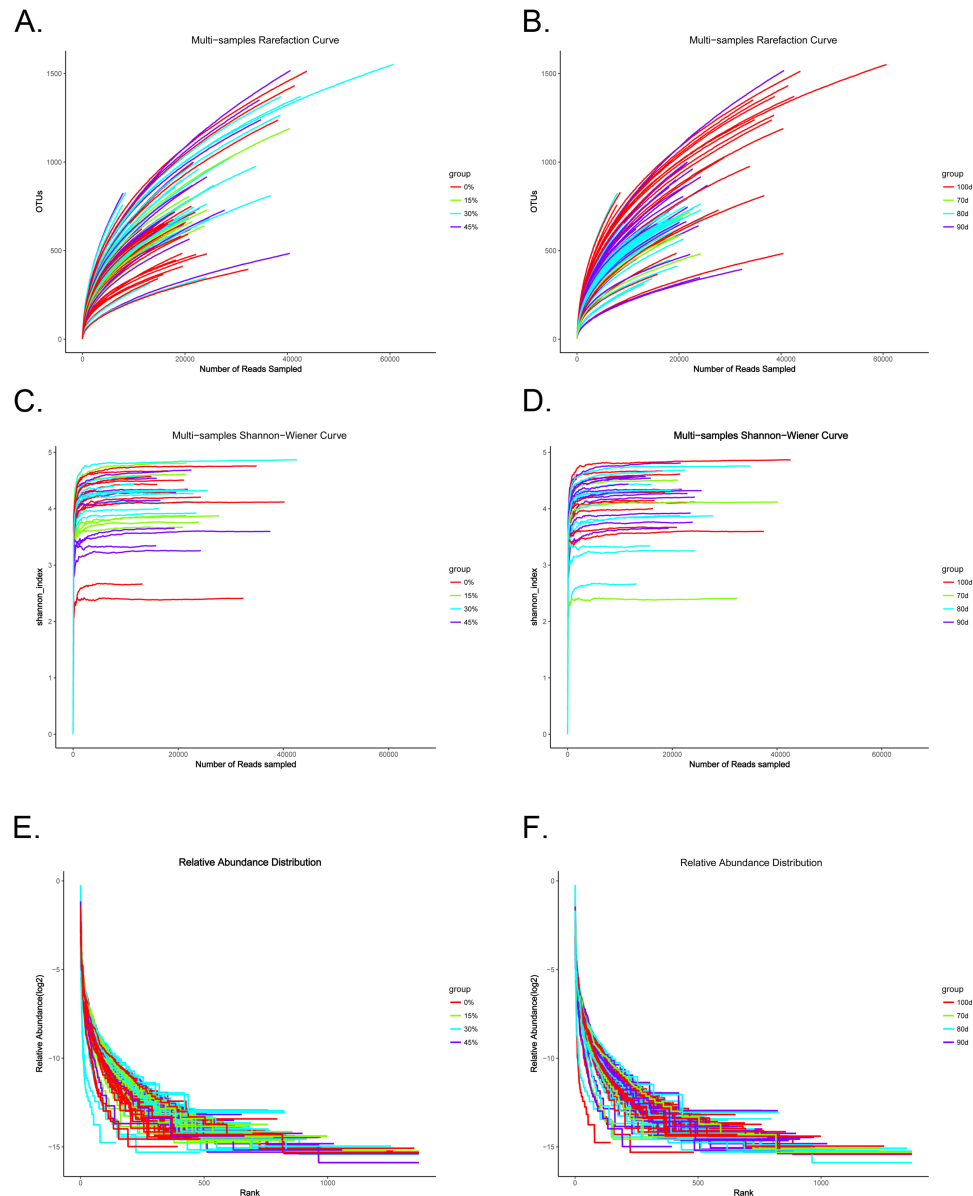


Fig 2. Alpha-diversity indices of the cecal bacterial community. Rarefaction analyses, based on 97% dissimilarity, were used to assess operational taxonomical units (OTUs) among groups with different (A) levels of green forage and (B) fattening duration. Shannon-Wiener analyses among groups with different (C) level of green forage and (D) fattening duration, and the abundance of microbiota among groups with different (E) levels of green forage and (F) fattening duration are shown.

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($P = 0.022$ and $P = 0.032$, respectively) than it was in samples from geese fattened for 20 and 30 days ($P = 0.019$ and $P = 0.030$, respectively).

The percentage of *Negativicutes* exhibited a strong negative linear relationship with green forage supplementation ($P = 0.029$, $R^2 = 0.942$), and the percentage in the cecal samples of geese fed a diet without green forage was significantly higher than that found in samples from geese fed 30 or 45% green forage ($P = 0.013$ and $P = 0.009$, respectively).

At the family level (Table 6 and S6D Fig), the most abundant microbial community was *Bacteroidaceae*, followed by *Prevotellaceae*, *Ruminococcaceae*, *Desulfovibrionaceae*, *Rikenellaceae*,

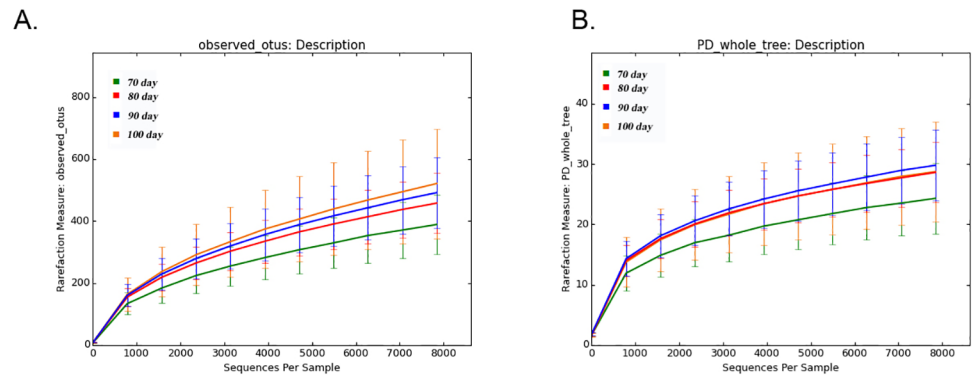


Fig 3. Effects of fattening duration on cecal microbiota diversity and composition. Rarefaction curves calculated at the lowest subsample size of 10,000 sequences per sample indicate the effects of sequencing efforts on the observed number of operational taxonomical units (OTUs) at (A) 97% sequence similarity and (B) phylogenetic diversity (based on the entire tree).

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Porphyromonadaceae, *Veillonellaceae*, and *Acidaminococcaceae*. The *Prevotellaceae* percentage was significantly different among fattening durations ($P < 0.0001$), with highest percentage in cecal samples from geese collected before fattening. However, the *Prevotellaceae* percentage exhibited no linear correlation with fattening duration ($P = 0.141$). The *Porphyromonadaceae* and *Ruminococcaceae* percentages exhibited strong positive correlations with fattening duration ($P = 0.045$, $R^2 = 0.912$ and $P = 0.009$, $R^2 = 0.982$, respectively). The *Porphyromonadaceae* percentage from the cecal samples of geese fattened for 30 days was significantly higher than that of samples collected before ($P = 0.001$) or after fattening for 10 days ($P = 0.014$). The *Ruminococcaceae* percentage was lower in geese before or after fattening for 10 days ($P < 0.0001$ and $P < 0.0001$, respectively) than in those fattened for 20 and 30 days ($P = 0.002$ and $P < 0.0001$, respectively). The *Bacteroidaceae* percentage was lower in geese before fattening than it was in geese fattened for 10 and 30 days ($P < 0.0001$ and $P = 0.0006$, respectively). The *Acidaminococcaceae* percentage was higher in geese fattened for 10 days than it was in geese fattened for 20 and 30 days ($P = 0.013$ and $P = 0.003$, respectively). The *Veillonellaceae* percentage exhibited a strong negative linear relationship with fattening time ($P = 0.044$, $R^2 = 0.914$), indicating that

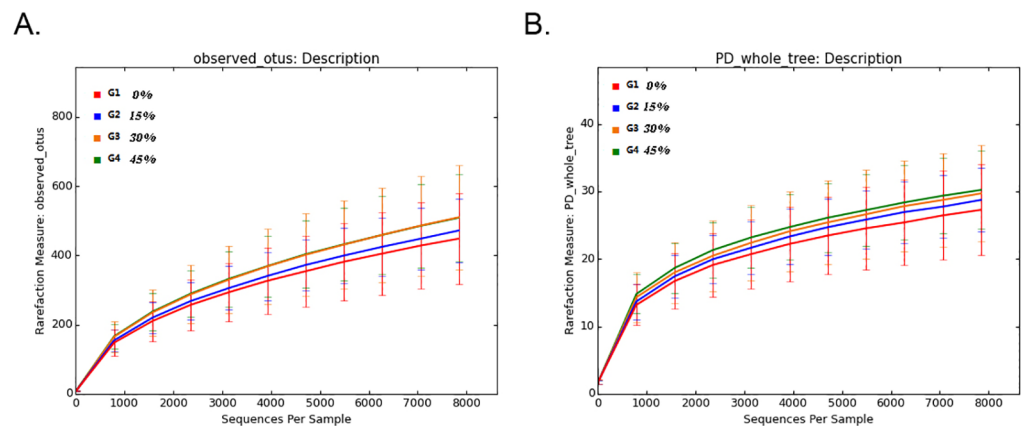


Fig 4. Effects of green forage inclusion on cecal microbiota diversity and composition. Green forage inclusion did not significantly affect the observed number of (A) operational taxonomical units (OTUs) and (B) phylogenetic diversity (based on the entire tree).

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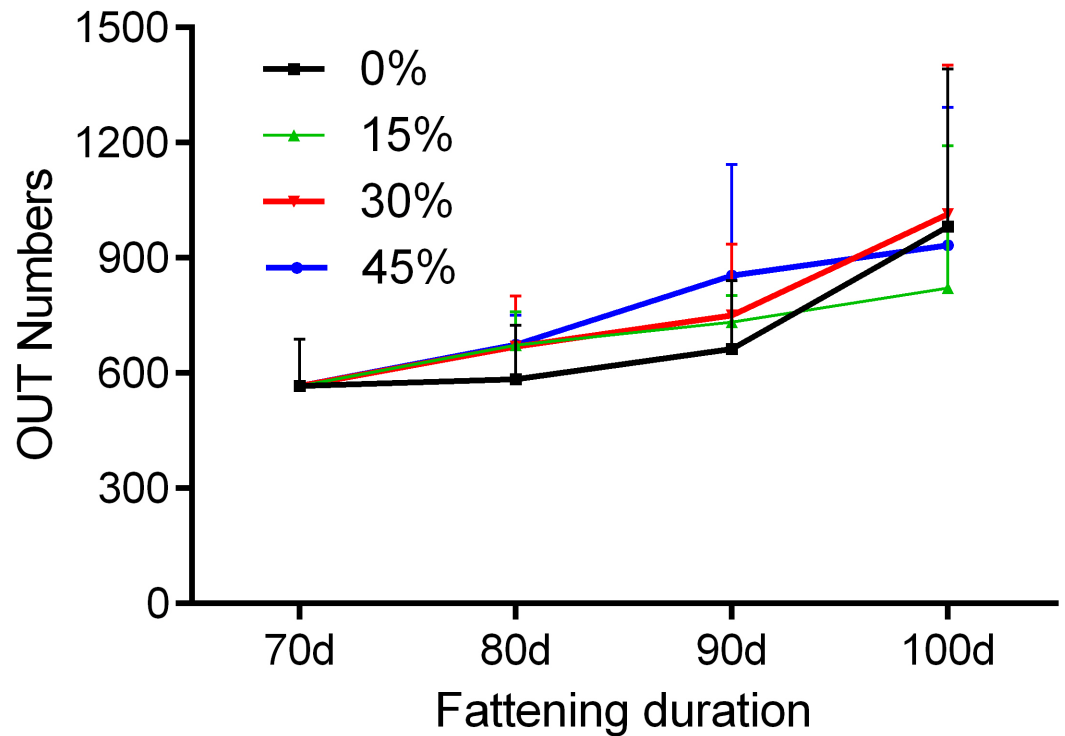


Fig 5. Comparison of cecal microbiota abundance in geese based on fattening durations and green forage inclusion levels. Data represent means ± standard deviation of microbiota based on the fattening duration (time) and green forage inclusion (colored line).

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Table 3. Abundances of the major phylum in each group and fattening time.

| Fattening duration | Green forage | <i>Deferribacteres</i> | <i>Firmicutes</i> | <i>Bacteroidetes</i> | <i>Spirochaetae</i> | <i>Proteobacteria</i> |
|---------------------------------|--------------|------------------------|-------------------|----------------------|---------------------|-----------------------|
| 0 (n = 8) | 0 | 1.17 | 18.53 | 71.58 | 0.88 | 7.82 |
| 10 (n = 36) | 0 | 0.82 | 19.07 | 74.12 | 0.95 | 4.78 |
| | 15 | 0.30 | 17.62 | 71.96 | 2.86 | 6.91 |
| | 30 | 0.63 | 19.03 | 69.98 | 2.51 | 7.59 |
| | 45 | 0.44 | 16.11 | 71.18 | 3.07 | 8.87 |
| 20 (n = 36) | 0 | 1.79 | 18.40 | 65.57 | 4.89 | 8.98 |
| | 15 | 0.27 | 19.12 | 71.00 | 1.90 | 7.40 |
| | 30 | 0.20 | 21.06 | 63.76 | 4.34 | 10.11 |
| | 45 | 0.33 | 21.08 | 66.72 | 2.43 | 8.77 |
| 30 (n = 36) | 0 | 0.38 | 22.72 | 68.08 | 1.38 | 7.27 |
| | 15 | 0.59 | 17.31 | 72.54 | 1.53 | 7.72 |
| | 30 | 0.91 | 21.50 | 64.55 | 3.65 | 9.09 |
| | 45 | 0.31 | 22.95 | 63.19 | 1.81 | 11.35 |
| SEM | | 0.024 | 1.477 | 2.808 | 0.198 | 1.348 |
| P value | | | | | | |
| Green forage | | 0.507 | 0.458 | 0.275 | 0.451 | 0.246 |
| Fattening duration | | 0.233 | 0.056 | 0.041 | 0.013 | 0.268 |
| Green forage×fattening duration | | 0.711 | 0.609 | 0.931 | 0.259 | 0.862 |

<https://doi.org/10.1371/journal.pone.0204210.t003>

Table 4. Abundances of the major orders based on groups and fattening times.

| Fattening duration | Green forage | <i>Clostridiales</i> | <i>Desulfovibrionales</i> | <i>Erysipelotrichales</i> | <i>Bacteroidales</i> | <i>Selenomonadales</i> | <i>Spirochaetales</i> |
|---------------------------------|--------------|----------------------|---------------------------|---------------------------|----------------------|------------------------|-----------------------|
| 0 (n = 8) | 0 | 10.59 | 9.06 | 1.26 | 69.63 | 8.76 | 0.69 |
| 10 (n = 36) | 0 | 8.83 | 4.86 | 0.93 | 74.64 | 9.41 | 1.35 |
| | 15 | 10.00 | 5.13 | 1.80 | 74.42 | 5.39 | 3.26 |
| | 30 | 13.96 | 5.36 | 1.13 | 71.65 | 5.03 | 2.86 |
| | 45 | 11.68 | 8.82 | 0.72 | 71.19 | 4.38 | 3.22 |
| 20 (n = 36) | 0 | 16.06 | 9.17 | 0.98 | 64.43 | 5.42 | 3.94 |
| | 15 | 12.80 | 5.43 | 1.01 | 71.33 | 6.95 | 2.48 |
| | 30 | 14.44 | 9.18 | 1.32 | 68.92 | 3.66 | 2.47 |
| | 45 | 18.32 | 8.44 | 1.11 | 65.21 | 3.33 | 3.59 |
| 30 (n = 36) | 0 | 15.07 | 3.80 | 0.74 | 74.67 | 4.17 | 1.54 |
| | 15 | 15.34 | 8.03 | 0.95 | 70.10 | 3.63 | 1.95 |
| | 30 | 18.27 | 7.15 | 0.65 | 68.63 | 2.08 | 3.21 |
| | 45 | 23.08 | 9.40 | 1.59 | 61.79 | 1.85 | 2.29 |
| SEM | | 0.445 | 0.391 | 0.348 | 3.845 | 0.575 | 0.531 |
| P value | | | | | | | |
| Green forage | | 0.103 | 0.204 | 0.921 | 0.227 | 0.024 | 0.355 |
| Fattening duration | | <0.0001 | 0.403 | 0.930 | 0.021 | <0.0001 | 0.001 |
| Green forage×fattening duration | | 0.751 | 0.583 | 0.466 | 0.870 | 0.110 | 0.204 |

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the highest percentage occurred in geese before fattening, while the lowest was observed in geese fattened for 30 days ($P < 0.0001$). The microbial communities did not differ among the green forage supplementation groups.

At the genus level (Table 7 and S6E Fig), the most abundant microbial community was *Bacteroides*, followed by *Prevotella* and *Desulfovibrio*. The *Prevotella* percentage was highest in

Table 5. Abundance of the major classes based on green forage fed groups and fattening times.

| Fattening time | Green forage | <i>Negativicutes</i> | <i>Deltaproteobacteria</i> | <i>Clostridia</i> | <i>Spirochaetes</i> | <i>Bacteroidia</i> | <i>Erysipelotrichia</i> |
|---------------------------------|--------------|----------------------|----------------------------|-------------------|---------------------|--------------------|-------------------------|
| 0 (n = 8) | 0 | 8.50 | 6.92 | 8.97 | 0.90 | 73.30 | 1.02 |
| 10 (n = 36) | 0 | 8.15 | 3.85 | 9.44 | 0.97 | 75.70 | 1.14 |
| | 15 | 5.46 | 6.31 | 10.80 | 2.90 | 72.92 | 1.27 |
| | 30 | 4.95 | 6.17 | 13.13 | 2.60 | 71.47 | 0.97 |
| | 45 | 3.91 | 8.11 | 11.64 | 3.12 | 72.17 | 0.67 |
| 20 (n = 36) | 0 | 4.19 | 7.92 | 13.81 | 5.01 | 67.45 | 0.91 |
| | 15 | 4.95 | 5.42 | 13.44 | 1.93 | 71.95 | 0.82 |
| | 30 | 2.67 | 9.11 | 16.92 | 4.42 | 64.76 | 1.76 |
| | 45 | 4.17 | 7.06 | 16.28 | 2.50 | 68.19 | 1.05 |
| 30 (n = 36) | 0 | 3.46 | 5.53 | 18.36 | 1.41 | 69.24 | 1.14 |
| | 15 | 3.18 | 7.21 | 13.43 | 1.55 | 73.47 | 0.91 |
| | 30 | 2.62 | 7.91 | 18.53 | 3.75 | 65.81 | 0.87 |
| | 45 | 1.52 | 10.74 | 20.15 | 1.84 | 64.05 | 1.45 |
| SEM | | 0.096 | 1.312 | 1.493 | 0.387 | 2.683 | 0.273 |
| P value | | | | | | | |
| Green forage | | 0.026 | 0.197 | 0.095 | 0.435 | 0.254 | 0.919 |
| Fattening duration | | <0.0001 | 0.404 | <0.0001 | 0.013 | 0.019 | 0.932 |
| Green forage×fattening duration | | 0.118 | 0.564 | 0.751 | 0.265 | 0.904 | 0.453 |

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Table 6. Abundance of the major families based on green forage fed groups and fattening times.

| Fattening time | Green forage | <i>Desulfotribionaceae</i> | <i>Prevotellaceae</i> | <i>Porphyromonadaceae</i> | <i>Bacteroidaceae</i> | <i>Acidaminococcaceae</i> | <i>Ruminococcaceae</i> | <i>Rikenellaceae</i> | <i>Veillonellaceae</i> |
|---------------------------------|--------------|----------------------------|-----------------------|---------------------------|-----------------------|---------------------------|------------------------|----------------------|------------------------|
| 0 (n = 8) | 0 | 7.15 | 31.29 | 3.78 ^b | 30.09 | 2.76 | 7.82 | 5.05 | 5.98 |
| 10 (n = 36) | 0 | 4.05 | 23.75 | 4.31 ^b | 42.94 | 3.68 | 8.03 | 3.80 | 4.86 |
| | 15 | 6.81 | 19.85 | 4.74 ^b | 38.48 | 3.27 | 9.00 | 7.49 | 2.59 |
| | 30 | 6.75 | 19.87 | 4.36 ^b | 40.96 | 2.18 | 10.39 | 5.71 | 3.13 |
| 20 (n = 36) | 45 | 8.85 | 17.70 | 5.29 ^b | 39.66 | 2.46 | 9.34 | 6.91 | 1.80 |
| | 0 | 9.18 | 22.27 | 5.61 ^b | 34.70 | 2.23 | 11.50 | 6.43 | 2.31 |
| | 15 | 5.88 | 30.01 | 4.92 ^b | 33.52 | 2.14 | 11.13 | 4.97 | 3.16 |
| 30 (n = 36) | 30 | 10.31 | 20.01 | 4.88 ^b | 30.53 | 2.21 | 13.84 | 9.19 | 0.71 |
| | 45 | 7.72 | 19.39 | 4.46 ^b | 36.92 | 1.99 | 13.73 | 5.92 | 2.49 |
| | 0 | 5.98 | 15.10 | 11.98 ^a | 38.50 | 1.99 | 15.37 | 5.12 | 1.72 |
| SEM | 15 | 7.78 | 20.97 | 3.81 ^b | 38.46 | 2.71 | 11.36 | 7.23 | 0.71 |
| | 30 | 8.92 | 15.11 | 6.00 ^b | 38.25 | 1.75 | 15.77 | 6.13 | 1.14 |
| | 45 | 11.73 | 12.27 | 5.40 ^b | 39.75 | 1.28 | 15.83 | 6.68 | 0.38 |
| | | 1.432 | 3.437 | 1.013 | 2.734 | 0.380 | 1.378 | 0.876 | 0.715 |
| P value | | | | | | | | | |
| Green forage | | 0.186 | 0.323 | 0.067 | 0.817 | 0.153 | 0.245 | 0.214 | 0.258 |
| Fattening duration | | 0.288 | <0.0001 | 0.011 | <0.0001 | 0.006 | <0.0001 | 0.175 | <0.0001 |
| Green forage×fattening duration | | 0.449 | 0.907 | 0.031 | 0.971 | 0.585 | 0.912 | 0.086 | 0.326 |

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Table 7. Abundance of the major genera based on green forage fed groups and fattening times.

| Fattening time | Green forage | RC9gutgroup | Alistipes | Parabacteroides | Prevotella | Bacteroides | Incertaesedis | Desulfovibrio | Megamonas | Phascolarctobacterium |
|---------------------------------|--------------|-------------|-----------|-----------------|------------|-------------|---------------|---------------|--------------------|-----------------------|
| 0 (n = 8) | 0 | 1.89 | 3.35 | 2.17 | 21.41 | 32.97 | 3.13 | 7.77 | 3.32 ^a | 3.04 |
| 10 (n = 36) | 0 | 1.68 | 2.38 | 3.26 | 12.62 | 45.44 | 2.90 | 4.34 | 3.35 ^a | 3.93 |
| | 15 | 4.09 | 3.24 | 3.14 | 8.13 | 40.01 | 3.06 | 7.05 | 1.67 ^{bc} | 3.41 |
| | 30 | 2.41 | 3.32 | 2.67 | 9.10 | 43.71 | 3.63 | 7.08 | 2.00 ^b | 2.33 |
| | 45 | 2.73 | 4.16 | 3.44 | 5.80 | 41.72 | 2.70 | 9.26 | 0.97 ^c | 2.57 |
| 20 (n = 36) | 0 | 3.78 | 2.36 | 2.78 | 12.93 | 35.74 | 3.05 | 9.09 | 1.06 ^{bc} | 2.30 |
| | 15 | 2.45 | 2.59 | 3.02 | 19.49 | 36.07 | 3.71 | 6.35 | 1.51 ^{bc} | 2.27 |
| | 30 | 3.82 | 4.91 | 2.97 | 8.66 | 31.17 | 3.41 | 10.38 | 0.54 ^c | 2.30 |
| | 45 | 2.35 | 3.64 | 3.27 | 8.69 | 39.23 | 2.94 | 8.13 | 1.42 ^{bc} | 2.11 |
| 30 (n = 36) | 0 | 2.20 | 3.57 | 4.12 | 5.05 | 44.35 | 7.34 | 6.87 | 1.66 ^{bc} | 2.26 |
| | 15 | 3.29 | 3.84 | 2.58 | 6.52 | 39.27 | 3.39 | 7.93 | 0.60 ^c | 2.77 |
| | 30 | 2.71 | 3.68 | 3.29 | 4.52 | 40.63 | 5.11 | 9.52 | 0.90 ^c | 1.85 |
| | 45 | 4.03 | 3.18 | 3.07 | 0.72 | 44.18 | 4.36 | 13.25 | 0.39 ^c | 1.41 |
| SEM | | 0.388 | 0.589 | 0.333 | 1.478 | 2.812 | 0.715 | 1.492 | 0.365 | 0.403 |
| P value | | | | | | | | | | |
| Green forage | | 0.827 | 0.250 | 0.524 | 0.394 | 0.525 | 0.450 | 0.142 | 0.042 | 0.159 |
| Fattening duration | | 0.147 | 0.947 | 0.001 | <0.0001 | <0.0001 | 0.006 | 0.236 | <0.0001 | 0.004 |
| Green forage×fattening duration | | 0.422 | 0.484 | 0.549 | 0.913 | 0.866 | 0.404 | 0.532 | 0.038 | 0.637 |

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cecal samples collected from geese before fattening compared to the examined durations ($P < 0.0001$). The percentage of *Bacteroides* was higher in samples collected from geese fattened for 10 or 30 days than it was in those collected from geese before or after fattening for 20 days ($P < 0.0001$). The percentage of *Oscillospira* was higher in geese fattened for 20 and 30 days than it was in geese before or after fattening for 10 days. The percentage of *Incertaesedis* was higher in geese fattened for 30 days than it was in geese fattened for the other durations ($P = 0.006$). The *Megamonas* percentage exhibited a strong negative relationship with fattening time ($P = 0.047$, $R^2 = 0.908$), and a higher percentage was detected in geese before fattening than in geese at the other three fattening durations ($P < 0.0001$). Furthermore, a higher percentage of *Megamonas* was detected in geese fattened for 10 days than in those fattened for 20 and 30 days ($P = 0.003$ and $P = 0.0004$, respectively). The percentage of *Phascolarctobacterium* was higher in geese before or after fattening for 10 days than in geese fattened for 20 and 30 days ($P = 0.004$).

The percentage of *Oscillospira* in the cecal samples of geese exhibited a strong positive linear relationship with green forage supplementation ($P = 0.023$, $R^2 = 0.954$). The percentage of *Oscillospira* in the cecal samples of geese fed a diet without green forage was lower than that of geese fed 30 and 45% green forage ($P = 0.009$ and $P = 0.002$, respectively). The percentage of *Megamonas* was higher in geese fed a diet without green forage than it was in geese fed diets with 30 and 45% green forage supplementation ($P = 0.024$ and $P = 0.008$, respectively). The interaction between green forage and fattening duration suggested that short fattening time and lower green forage inclusion resulted in higher percentage of *Megamonas* (Table 7).

3.6. Fatty acid content in breast muscle of Wanxi white geese fed different level of green forage for different fattening duration

Linoleic acid (C18:2) was lower in geese before fattening as compared with other time points with or without green forage inclusion ($P < 0.05$, Table 8). Geese fed 15% green forage and fattening for 30 days exhibited higher C18:2 as compared with others ($P < 0.05$). Linolenic acid (C18:3) was higher in geese fed diet with 30–45% green forage inclusion and fattening for 20–

Table 8. Fatty acid content in geese fed different level of green forage for different fattening duration (%).

| Fattening duration | Green forage | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C18:3 | C20:3 |
|--------------------|--------------|-------|-------|-------|-------|---------------------|--------------------|-------|
| 0 (n = 8) | 0% | 20.09 | 1.01 | 18.72 | 26.78 | 17.28 ^d | 0.25 ^{bc} | 13.30 |
| 10 (n = 36) | 0% | 21.98 | 1.11 | 18.52 | 23.77 | 18.14 ^{bc} | 0.15 ^c | 13.04 |
| | 15% | 20.42 | 1.05 | 18.89 | 25.31 | 17.59 ^{cd} | 0.20 ^{bc} | 13.15 |
| | 30% | 22.36 | 1.26 | 17.27 | 25.04 | 17.87 ^{bc} | 0.28 ^{bc} | 12.53 |
| | 45% | 22.11 | 1.04 | 17.84 | 24.11 | 18.65 ^{ab} | 0.30 ^{bc} | 12.47 |
| 20 (n = 36) | 0% | 23.07 | 1.21 | 16.73 | 25.62 | 18.10 ^{bc} | 0.36 ^b | 11.69 |
| | 15% | 23.79 | 1.52 | 14.54 | 29.04 | 17.59 ^{cd} | 0.51 ^a | 9.75 |
| | 30% | 21.63 | 1.28 | 16.80 | 25.80 | 18.60 ^{ab} | 0.41 ^{ab} | 12.21 |
| | 45% | 22.90 | 1.49 | 16.20 | 26.30 | 18.14 ^{bc} | 0.48 ^a | 12.04 |
| 30 (n = 36) | 0% | 21.68 | 1.81 | 15.52 | 27.16 | 19.44 ^a | 0.35 ^b | 11.25 |
| | 15% | 23.30 | 1.56 | 14.48 | 27.98 | 19.51 ^a | 0.49 ^a | 9.81 |
| | 30% | 23.63 | 2.03 | 13.70 | 29.41 | 18.47 ^{ab} | 0.57 ^a | 9.34 |
| | 45% | 23.37 | 1.88 | 14.23 | 28.35 | 18.88 ^{ab} | 0.55 ^a | 9.91 |
| SEM | | 0.24 | 0.05 | 0.26 | 0.42 | 0.13 | 0.02 | 0.27 |
| P | | 0.156 | 0.326 | 0.284 | 0.837 | 0.050 | 0.017 | 0.541 |

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30 days as compared with geese before fattening, fattening for 10 days, or fattening with 15% green forage inclusion in diet ($P < 0.05$, Table 8).

3.7. Canonical correspondence analysis (CCA) between microbiota and FA component in breast muscle

Canonical correspondence analysis (CCA) is a multivariate extension of weighted averaging ordination, which is a simple method used to arrange microbial communities based on environmental variables. CCA was used to rank microbial communities based on their importance in determining FA composition. The ordination diagram generated using CCA visualizes the main features of microbial community distribution based on FA variables as well as the importance of those variables. The CCA ordination diagram is constructed and interpreted as follows (Fig 6): the arrows represent the FA variables; the coordinates of the arrows are the values of the arrows on the two best synthetic gradients (axes 1 and 2 in Fig 6); the direction of the arrows represents the maximum change of the associated variables; the length of an arrow representing a FA variable is equal to the rate of change in the weighted averaging ordination, and is a measure of how much the microbial communities differ with regard to the FA variables.

Important FAs tend to be represented by longer arrows than less important ones. According to Fig 6, *Bacteroides* correlated with nearly all detected FAs, and the greatest correlations were associated with eicosatrienoic acid (C20:3), palmitic acid (C16:0), and oleic acid (C18:1). These results suggest that percentage of C20:3, C16:0, and C18:1 could be increased when geese are fattened 10–30 days. *Megamonas*, *Phascolarctobacterium*, RC9gutgroup, and *Parabacteroides* were all set near the reverse extension line of linolenic acid (C18:3), while others such as *Treponema*, *Desulfovibrio*, and *Prevotella* were set along the line of C18:1.

4. Discussion

In China, geese reproduction exhibits strong seasonal adaptation, and the birds usually lay eggs during winter and early spring. The eggsthen hatchin the spring, and goose fattening is performed when the birds areapproximately 60–70 days old duringthe summer. Geese are herbivorous, so green forage was suggested to be includedto reduce feed conversion ratio and

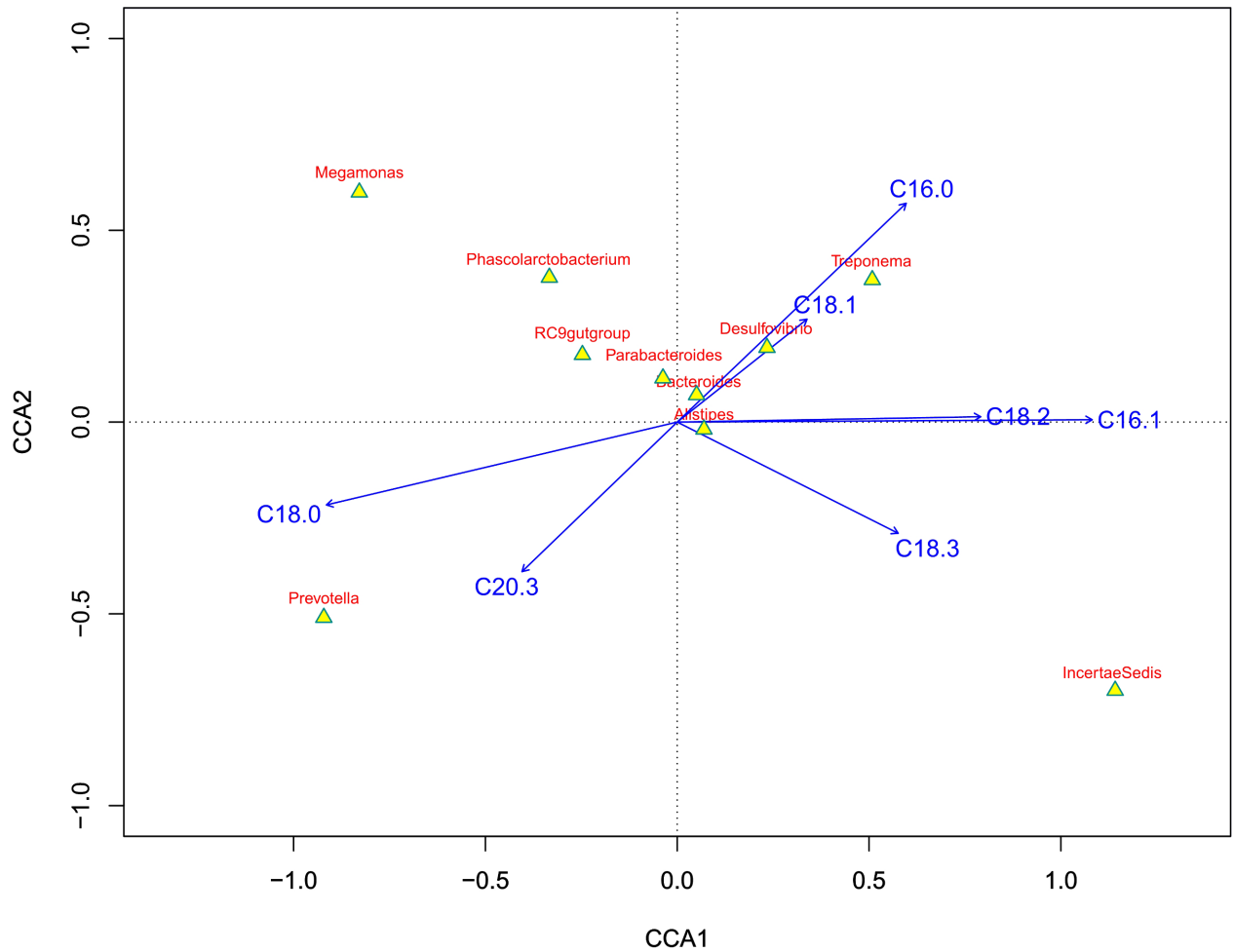


Fig 6. Canonical correspondence analysis (CCA) of the relationship between the relative abundance of bacteria (phylum level) and the percentage of muscle fatty acid (FA, environmental variables). Microbial phyla are shown as yellow triangles, and muscle FAs are shown as blue arrows. Microbial phyla can be vertically projected along the ray elongated from the blue arrows. The abundance was measured as the relative distance from the projection to the arrow, and the distance along the direction of the arrow was considered an increase (and vice versa). Acute and obtuse angles between the environmental variables and phyla were considered positive and negative correlations, respectively.

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promote health [6]. In China, the most commonly available green forage during summer is white cabbage, and reports suggest that green forage could regulate intestinal microflora, thereby improving feed utilization [6,13].

Although no difference was observed in bacterial communities, our results indicated that green forage inclusion and fattening duration could numerically increase α -diversity and PD. These findings are highly in accordance with those of Wang et al. [14], which reported that green forage or crude fiber could enrich intestinal microflora.

Ruminants can digest crude fiber because they possess ruminal and cecal microbes [15]. However, in monogastric animals, the digestion of cellulose and hemicelluloses depends solely on colon and cecal bacteria. Microbiota that can digest crude fiber mainly inhabit the cecum, which is the most important site for feed fermentation in geese. Volatile FAs in the cecum are the final products of carbohydrate decomposition. Furthermore, the activity of cecal microbiota in animals could be influenced by factors such as feed and age [9,10]. Green forage

inclusion is thought to enhance microbial activity in the cecum, thus promoting cellulose digestion in geese.

The cecal microflora of geese was mainly influenced by the fattening duration, and this result is consistent with the findings of Pourabedin et al. [10], which reported that gut microbiota varied in chickens at different ages. Ban-Tokuda et al. [16] suggested that the level of *Bacteroidetes* correlated with body weight and feed intake, and it decreased with fattening. This suggests that fattening geese for 10 days might result in higher feed intake than fattening for other durations. The members of *Firmicutes* and *Bacteroidetes* phyla dominate microbiota of animals [17,18], and lean animals are thought to have higher and lower levels of *Bacteroidetes* and *Firmicutes*, respectively, among gut microbes [17]. Although more polyunsaturated FAs are produced in geese fattened for a longer time [12], the meat production performance does not seem to be ameliorated based on the high percentages of *Bacteroidetes* from the ceca microbiota. Proper green forage inclusion (15%) might enhance fat deposition, whereas 30–45% inclusion could decrease fat deposition.

Inigo et al. [19] reported that rabbits fed a diet containing glycerol showed an increased *Clostridiales* percentage during fattening. Many studies reported that green forage inclusion could promote FA synthesis in animals [20, 21], this might suggest that the increased *Clostridiales* percentage could accelerate FA synthesis, especially that of UFAs. The *Selenomonadales* order belongs to the *Firmicutes* phylum, and studies in mice demonstrated that the abundance of *Firmicutes* is proportionate to obesity levels, high efficiency of FA absorption, and fat deposits [17,22–23]. This suggests that green forage inclusion could alleviate fat deposition in geese. Walugembe et al. [24] stated that broilers fed a high fiber diet showed an increase in the relative abundance of the *Selenomonadales* order, and this result differs greatly from the results of our experiment. Cecal microbial communities differ considerably among poultry breeds [24], and they are mainly responsible for fermentable nutrient availability and FA synthesis [14]. The decreased abundance of *Selenomonadales* (class *Negativicutes*) in geese fed diet with high percentage of green forage or subjected to fattening for long period indicates that this microbiota might play a minor role in FA synthesis, and *Helicobacter* and *Megamonas* genera exhibited increased abundance.

It is known that *Prevotella* degrades readily metabolizable carbohydrates, and it is more abundant in low-fiber diets [25]. These data are consistent with our experimental results in that a higher abundance of *Prevotellaceae* was detected in geese before than after fattening, and a lower percentage of UFA was detected during this stage than at other stages. Putative taxonomic identifications have linked *Bacteroidia* and *Porphyromonadaceae* (unclassified *Bacteroidales*) strains with C18:1 as well as *cis*-9 and *trans*-11 conjugated linoleic acid concentrations in the rumen of cattle and sheep [26, 27]. These identifications explain why the highest *Porphyromonadaceae* and *Ruminococcaceae* percentages were detected in geese fattened for 10–30 days, and this might indicate that appropriate fattening durations should be used for geese. Increased levels of *Bacteroidia*, *Porphyromonadaceae*, and *Ruminococcaceae* in ceca likely reflects the need for fiber digestion in geese. A higher fiber intake than usual might significantly increase the abundance of *Acidaminococcaceae* in wild turkeys [28], and this is consistent with the results of our study in that a higher abundance of *Acidaminococcaceae* was detected in geese fed green forage or those that had been fattened.

Bacteroides and *Prevotella* were collectively numerically dominant in the rumen of cows [29], and these genera might be responsible for nutrient utilization. Fiber inclusion might inhibit nutrient utilization, thus decreasing *Prevotella* in the ceca of geese. As described by Ghaffarzadegan et al. [30], the addition of fibers from guar gum and pectin could decrease the cecal abundance of *Oscillospira* and an unclassified genus in the *Ruminococcaceae* family.

Green forage inclusion could increase *Oscillospira* abundance, which might indicate that fiber type could modulate cecal microbiota composition.

An increased abundance of *Megamonas* was detected in broilers fed a high fiber diet [24], and these results are in contrast to our present results. Considering the function of the ceca in broilers and geese, it is clear that the ceca is very small in broilers and responsible for water absorption; however, it is important for fiber digestion in geese. Dietary fiber is usually limited to less than 5% in broilers, whereas more than 10% is suggested in geese.

Phascolarctobacterium plays a critical role in the dynamic balance of gut microbiota, and it could be responsible for the production of short-chain FAs [31]. Essential FAs such as linoleic acid and alpha-linolenic acid cannot be synthesized *de novo*. Therefore, the lower content of short-chain FAs might indicate a higher level of long chain UFAs and a lower abundance of *Phascolarctobacterium* in geese fed 30–45% green forage.

5. Conclusion

This study demonstrated that green forage inclusion and the fattening duration increased the abundance of cecal microflora in geese. The bacterial community was mainly influenced by the fattening duration, and *Bacteroidales* and *Selenomonadales* orders and *Megamonas* and *Oscillospira* genera were affected by green forage inclusion levels. Finally, *Bacteroidales* might represent an intestinal promoter that improves UFA synthesis.

Supporting information

S1 Fig. Relative abundances (% of total sequences) of the most abundant bacterial phyla identified based on Ion Personal Genome Machine (PGM) sequencing of the cecal contents of geese fed diets with different levels of green forage for fattening at different durations. (JPG)

S2 Fig. Relative abundances (% of total sequences) of the most abundant bacterial classes identified based on Ion Personal Genome Machine (PGM) sequencing of the cecal contents of geese fed diets with different levels of green forage for fattening at different durations. (JPG)

S3 Fig. Relative abundances (% of total sequences) of the most abundant bacterial orders identified based on Ion Personal Genome Machine (PGM) sequencing of the cecal contents of geese fed diets with different levels of green forage for fattening at different durations. (JPG)

S4 Fig. Relative abundances (% of total sequences) of the most abundant bacterial family identified based on Ion Personal Genome Machine (PGM) sequencing of the cecal contents of geese fed diets with different levels of green forage for fattening at different durations. (JPG)

S5 Fig. Relative abundances (% of total sequences) of the most abundant bacterial genus identified based on Ion Personal Genome Machine (PGM) sequencing of the cecal contents of geese fed diets with different levels of green forage for fattening at different durations. (JPG)

S6 Fig. Microbial community structures at each taxonomic level. Dendrogram illustrating bacterial community similarity based on (A) phylum, (B) order, (C) class, (D) family, and (E) genus. (PDF)

S1 Table. Major components and nutrition levels of the experimental diets.

(XLS)

S2 Table. Total number of reads and valid reads from the original FASTQ files associated with each sample. Valid reads (fragments) were obtained after short and mismatched sequences were removed. Valid ratio = Valid reads/Raw reads (%).

(XLS)

S3 Table. Most abundant bacterial communities at phylum, class, order, family, and genus levels in geese fed diets with different levels of green forage for fattening at different durations.

(XLSX)

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