Impact of the gut microecology on Campylobacter presence revealed by comparisons of the gut microbiota from chickens raised on litter or in individual cages

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Abstract

Background: Poultry is the major reservoir of Campylobacter that contributes to human campylobacteriosis and threatens food safety. Litter contact has been linked to Campylobacter colonization, but the gut microecological impact underlying this link remains not fully clear. Here, we sought to investigate the impact of the gut microecology on the presence of Campylobacter by examining the microbiota in the duodenum, jejunum, ileum, ceca, and feces from chickens raised on commercial litter and in individual cages at 0–57 days of age.

Results: Through litter contact, the presence of Campylobacter was found to benefit from microecological competition among Lactobacillus, Helicobacter, and genera that are halotolerant and aerobic or facultatively anaerobic in the upper intestine, such as Corynebacterium and Brachybacterium. The presence was also promoted by the increased abundance in obligate anaerobic fermentation microbes, especially members of the orders Clostridiales and Bacteroidales. The longitudinal analysis supported the vertical or pseudo-vertical transmission but suggested that colonization might occur immensely at 7–28 days of age. We observed a host genetic effect on the gut microecology, which might lead to increased heterogeneity of the microecological impact on Campylobacter colonization.

Conclusions: The findings advance the understanding of the gut microecological impact on Campylobacter presence in the chicken gut under conditions of litter contact and suggest that manipulations of the gut microecology, as well as the microbes identified in the Campylobacter association networks, might be important for the development of intervention strategies.

Keywords: Campylobacter, Chicken, Gut microbiome, Gut microecology

Background

Despite considerable global efforts, campylobacteriosis is still one of the most commonly reported foodborne infections in both developed [1, 2] and developing countries [3]. Poultry is known as the major reservoir of Campylobacter and the consumption of contaminated chicken meat is considered the main cause of...
human campylobacteriosis [1]. Therefore, on-farm control is important in campylobacteriosis intervention. To reduce Campylobacter in the chicken gut with the aim of diminishing the burden of campylobacteriosis, strict hygiene measures and biosecurity [4], and different feed additives, such as organic acids [4] and probiotics [5], have been used. Other potential strategies, such as bacteriophages [5], vaccines [6] and anti-Campylobacter bacteriocins [7], have also been proposed. However, Campylobacter colonization and prevalence are not yet well controlled [1].

The production environment is found to have impacts on Campylobacter colonization and prevalence. In an early study, higher isolation rates of Campylobacter jejuni were observed in broilers raised on litter than in those raised in cages [8]. While litter conditions have been reported to have no effect on Campylobacter colonization [9], studies have shown that chickens can be colonized by Campylobacter from contaminated litter through the fecal-oral route [10, 11]. These results indicate that contact between chickens and the litter would promote the development of Campylobacter colonization and its prevalence. Since Campylobacter survives within a complex gut microbial ecosystem instead of existing alone in the chicken gut, the interactions among microbes in the gut microecology should play an important role in Campylobacter colonization. However, how the gut microecology impacts the presence of Campylobacter in the chicken gut when in contact with litter remains not fully clear.

Increasing discoveries and perspectives on the gut microbiome have been reported from an ecological view [12–14], which requires as many taxa as possible to be examined in one study. Many previous studies on Campylobacter could examine only one or a few microorganisms with culture-dependent or low-throughput technologies [8, 15–17], which limited the identification of ecological relationships among microbes. Culture-independent high-throughput sequencing tools have brought new insights into understanding of microbial ecosystems. Through the use of high throughput sequencing technologies, chickens with high Campylobacter loads were observed to have increased gut microbial diversity [18–20] and an increased abundance of microbes, such as Clostridium [19, 21, 22] and Lachnospiraceae [19, 23]. However, the samples used in these studies were only from the ileum, ceca, or feces. While Campylobacter is enriched in the lower intestine instead of in the duodenum or jejunum, the interaction among microbes in the upper intestine might have an impact on the microbial composition as well as Campylobacter colonization in the lower intestine. Therefore, a comprehensive gut microecology covering the microflora from the upper and lower intestine as well as the feces is required to understand the role of the gut microecology on Campylobacter presence.

Most investigations and strategies proposed have focused on horizontal transmission of Campylobacter, and some studies have reported the lack of evidence for vertical transmission [15, 24, 25]. While studies have observed Campylobacter jejuni penetration through eggshells [26] and colonization in the egg contents [27], the vertical or pseudo-vertical transmission of Campylobacter has received little attention [27]. Thus, further study is needed to better understand the vertical or pseudo-vertical transmission of Campylobacter in chickens.

Host genetics have been largely observed to have impacts on the composition of the gut microbiota in humans [28–31]. A recent study revealed the role of the host’s genetics in manipulating fat deposition in chickens [32]. Therefore, the host genetic effect should be considered when investigating the gut microecology underlying the presence of Campylobacter resulting from litter contact.

Here, we took advantage of high-throughput sequencing technology to identify the gut microecological impact on Campylobacter presence in the chicken gut by comparing the gut microbiota from chickens raised on commercial litter with that in chickens raised in individual cages. Parents with pedigrees from a Campylobacter-positive population were used to generate the experimental chicks. We evenly allocated full and half-sib chicks from each family to two groups so that the chicks in the two groups had the same genetic and hatching environment background. The gut spatial and longitudinal analysis of the microbiota showed that Campylobacter colonization might benefit from microecological competition among Lactobacillus, Helicobacter, and genera that are halotolerant and aerobic or facultatively anaerobic and might be promoted by an increased abundance of obligate anaerobic fermentation microbes, especially members within the orders Clostridiales and Bacteroidales. Campylobacter colonization might commence at or before hatching, but Campylobacter could hardly colonize the chicks’ gut in the first week of age and may immensely colonize from 7 to 28 days of age. In addition, Campylobacter colonization is likely to be influenced by host genetics through the impact on the gut microecological composition.

**Results**

**Longitudinal and gut spatial dynamics of the Campylobacter abundance**

To investigate the microecological networks promoting Campylobacter colonization in the chicken gut, we examined the gut microbiota of chickens raised on commercial litter and used the gut microbiota of chickens raised in individual cages that are able to avoid fecal contact and
cross contamination as the comparison group (Supplementary Table 1). The Campylobacter-positive population was used as the parent generation to generate the experimental chicks. Full- and half-sib chicks from each family were evenly allocated to two groups so that the subjects in the two groups shared the same genetic and hatching environment background.

To characterize the Campylobacter presence, we examined the Campylobacter abundance using 16S rRNA gene sequencing with samples from 0 to 57 days of age in the two groups (Fig. 1A). Most chick samples at hatch were identified as having Campylobacter presence, including 98.3% of meconium, 76.9% of ileal and 96.2% of cecal samples. The Campylobacter abundance in both groups decreased from 0 to 7 days of age. At 7 days of age, Campylobacter was not detected in 70.0% of subjects in the litter group and in 96.7% of the cage group. However, the Campylobacter abundance in the litter group increased from 7 to 28 days of age and slowly decreased from 42 to 57 days of age, while no significant increase in abundance was observed in the cage group from 7 to 57 days of age. The longitudinal analysis suggests that Campylobacter could be carried by chicks through vertical/pseudo-vertical transmission, but Campylobacter could hardly colonize the chick gut or would be excreted out during the first week of life regardless of whether the chicks contacted the litter and suggests that intense colonization may occur from 7 to 28 days of age.

To understand the abundance of Campylobacter in both the upper and lower intestines in the two groups, we examined the Campylobacter abundance in the duodenum, jejunum, ileum, and feces (Fig. 1B). As expected, higher Campylobacter abundance was detected in the lower intestine than in the other sites. Since ceca are frequently examined in Campylobacter studies, we performed real-time quantitative PCR for cecal Campylobacter spp. and observed similar results as those from the high-throughput sequencing in which the Campylobacter abundance in the litter group was significantly higher than that in the cage group (Fig. 1C), suggesting that litter contact may promote the presence of Campylobacter in the chicken gut. Moreover, the comparative analysis suggests that the gut microbial contrast between chickens raised on conventional litter and those in individual cages is likely to be a good model to study Campylobacter colonization.

Characterization of the gut microflora and its relationship with Campylobacter dynamics

To investigate the impact of gut microbiology on the longitudinal dynamics of the Campylobacter abundance, we examined changes in gut microbial community over time in the litter and cage groups. The gut microbial community in the two groups significantly differentiated starting from 7 days of age (Fig. 2A), while the abundant microbes at the phylum (Fig. 2B) or genus (Supplementary Fig. 1) level varied between the two groups from as early as 2 days of age. Although both groups were dominated by Firmicutes in the whole experimental period, other abundant phyla varied over time in each group. Notably, the phylum Actinobacteria increased in abundance to become an abundant phylum in the litter group from 42 days of age (Fig. 2B). The increased abundance of this phylum was mainly due to genera that were found to be halotolerant and aerobic or facultative anaerobic, such as Brachybacterium, Brevibacterium, and Corynebacterium (Supplementary Fig. 1). Except for Lactobacillus that dominated the gut microflora in both groups from 7 days of age, the majority of abundant microbes varied in taxonomy and abundance between the two groups. For example, the genera Staphylococcus and Streptococcus as well as the halotolerant and aerobic or facultative anaerobic genera, such as Brachybacterium, Brevibacterium, Corynebacterium, Romboutsia, Faecalibacterium, C. parvus, uncharacterized Ruminococcaceae genus, Ruminococcaceae UCG−014, uncharacterized Lachnospiraceae genus, and Alistipes were more abundant in the cage group at the corresponding days of age (log10(LDA score) > 3.5; Fig. 2D and Supplementary Fig. 1). The results suggest that the increased abundances of the phylum Actinobacteria and the genera that are halotolerant and aerobic or facultative anaerobic largely contribute to the microecological differences and might contribute to the Campylobacter presence.

We examined the abundant and significantly differential microbes in samples from different gut sites to investigate the impact of the gut spatial microflora on Campylobacter dynamics. The phylum Firmicutes and genus Lactobacillus were found to be the dominant taxa at all examined gut sites in both the litter and cage groups at the phylum and genus levels, respectively (Fig. 2C; Supplementary Fig. 2). The genus Helicobacter, sharing a similar taxonomic lineage with Campylobacter within the phylum Epsilonbacteriota and order Campylobacterales, was found to be one of the most abundant microbes of the upper intestinal microflora in the litter group and was significantly higher in abundance than that in the cage group (log10(LDA score) > 3.5; Fig. 2E). In addition, the abovementioned halotolerant and aerobic or facultative anaerobic genera were also more abundant in the upper intestine in the litter group than in the cage group (log10(LDA score) > 3.5; Fig. 2E). Although the cecal microflora in both groups were dominated by similar microbes, such as Alistipes, Faecalibacterium,
Fig. 1 Longitudinal and gut spatial dynamics of the *Campylobacter* abundance. **A:** Relative abundance of *Campylobacter* in the litter and cage groups at different days of age as well as the *Campylobacter* abundance in parents’ fecal samples. **B:** Relative abundance of *Campylobacter* in the litter and cage groups in different gut sites at 57 days of age. D, J, I, C, and F denote the duodenum, jejunum, ileum, cecum, and feces, respectively. **C:** Quantitative changes in *Campylobacter* spp. in cecal samples by qPCR. One-way ANOVA was used to examine the significant difference between groups. ***P* < 0.001
Lactobacillus, Ruminococcaceae UCG-14, uncharacterized Lachnospiraceae genus, and uncharacterized Clostridiales vadin BB60 group, the abundances of these microbes were significantly different between the two groups (log10(LDA score) > 3.5; Fig. 2E). Within these microbes, such as Bacteroides, Barnesiella, Butyricimonas, Fecalibacterium, Oscillibacter, Phascolarctobacterium, and Megamonas, obligate anaerobic fermentation microbes were found to be more abundant in the hindgut of the litter group than in the cage group (log10(LDA score) > 3.5).

Most of these significantly differential microbes in ceca were short-chain fatty acid (SCFA)-producing bacteria and were mainly from the orders Bacteroidales and Clostridiales. To ensure that these bacteria were actively producing SCFAs in the hindgut, we measured theecal SCFAs, including formate, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate, and found that all measured SCFAs were significantly or marginally higher in the litter group than in the cage group (P < 0.05; acetate: P < 0.1), except for butyrate and valerate (P > 0.1) (Fig. 3). Higher SCFAs levels support the enrichment of SCFA-producing bacteria and more SCFA-producing activities in the hindgut of the litter group. Moreover, higher SCFAs might be linked to the higher abundance of Campylobacter as studies found that Campylobacter may benefit from the anaerobic fermentation by consuming SCFAs [33, 34].

Therefore, both compositional and differential analysis suggest that Helicobacter and genera that are halotolerant and aerobic or facultative anaerobic in the small intestine as well as obligate anaerobic fermentation microbes in the lower intestine might contribute to the Campylobacter presence in the chicken gut.

Gut microecological networks underlying Campylobacter presence

To further characterize how the gut microbiome promotes the presence of Campylobacter, we constructed microbial networks based on correlation analysis for the microbes at each of the examined gut sites (Fig. 4). In total, 115 genera, including 114 bacteria and 1 archaeon (genus Methanobrevibacter), showed relationships with Campylobacter (Supplementary Fig. 3). However, most of the identified relationships were positive while only three bacteria (Corynebacterium_1, Dietzia, and Staphylococcus) showed negative associations with Campylobacter (Fig. 4A, C and Supplementary Fig. 3). All three bacteria were found to be halotolerant and aerobic or facultative anaerobic genera. Other bacteria associated with Campylobacter were mainly from the orders Clostridiales and Bacteroidales. Of these bacteria, 53 were from Clostridiales (30 in Ruminococcaceae, 15 in Lachnospiraceae and 8 in others) and 14 were from Bacteroidales (3 in Prevotellaceae, 3 in Rikenellaceae, 3 in Barnesiellaceae, 2 in Marinilaceae, and 3 in others) (Fig. 4, Supplementary Fig. 3, and Supplementary Table 2). The abundance of these bacteria accounted for 60.4% of the genera associated with Campylobacter. Interestingly, although Campylobacter was found to be enriched in the ceca, it was positively associated with more microbes in the small intestine (63, 24, and 69 microbes in the duodenum, jejunum, and ileum, respectively) than in the ceca (8 microbes) (Fig. 4 and Supplementary Fig. 3). In addition, seven microbes (Barnesiella, Lachnospiraceae_UCG-010, Fournierella, Oscillibacter, Ruminoclostridium_9, Ruminococcaceae_UCG-007, and Megamonas) were positively associated with Campylobacter in all examined small intestinal sites with five of them belonging to the order Clostridiales. These results suggest that obligate anaerobic fermentation microbes, especially members within the order Clostridiales in both the upper and lower intestines, were associated with the Campylobacter presence.

Nonlinear microecological relationships appeared to play important roles in Campylobacter colonization as well. Lactobacillus and many halotolerant and aerobic or facultative anaerobic genera showed negative relationships with Campylobacter, but the relationships were not linear (Fig. 5). For example, when the abundance of Lactobacillus was over 10%, the Campylobacter abundance was lower than 0.5% in most samples (Fig. 5A). When Lactobacillus abundance was over 30%, the Campylobacter abundance in most samples was lower than 0.05% (Fig. 5B). Although Campylobacter still showed a negative relationship trend with Lactobacillus when the Campylobacter abundance was lower...
Fig. 2 (See legend on previous page.)
than 0.05%, the susceptibility of Campylobacter to Lactobacillus decreased (Fig. 5C). This result suggests that Lactobacillus has good potential to largely suppress Campylobacter colonization but could not thoroughly clear it. Similar results were also observed between Campylobacter and the halotolerant and aerobic or facultative anaerobic genera in the small intestine (Supplementary Fig. 4), which suggested that the halotolerant and aerobic or facultative anaerobic genera might also play an important role in the disruption of Campylobacter colonization.

However, the increase in Helicobacter is likely to mitigate the disruption of Campylobacter. Helicobacter showed a negative relationship with Lactobacillus (Fig. 4 and Supplementary Table 3), which might ameliorate the suppression of Campylobacter with the increase in Helicobacter abundance. Moreover, nonlinear relationships were observed between Helicobacter and genera that are halotolerant and aerobic or facultative anaerobic (Fig. 5D and Supplementary Fig. 5). The relationships between these microbes were found to shift from positive to negative with increasing Helicobacter abundance. For instance, Helicobacter showed a synergistic relationship with Corynebacterium_1 when Helicobacter abundance was lower than 30%, but the relationship shifted to become antagonistic when the abundance of Helicobacter was over 30%. The abundance of Corynebacterium_1 even decreased to close to zero when the abundance of Helicobacter was over 50%. Notably, the genera that are halotolerant and aerobic or facultative anaerobic also showed negative relationships with Lactobacillus (Fig. 4 and Supplementary Table 3), which might further mitigate the disruption of Campylobacter colonization. The results suggest that although Lactobacillus and genera that are halotolerant and aerobic or facultative anaerobic might disrupt Campylobacter colonization in the chicken gut, the microecological competition among these microbes and Helicobacter might mitigate the disruption.

Impact of litter contact and host genetics on the gut microecological uniformity

We next examined the microbial similarity within each group based on interindividual UniFrac distances to investigate to what extent litter contact and host’s genetics could affect the uniformity of the microbial community, which might subsequently influence the spread of Campylobacter in the population (Fig. 6). The average unweighted UniFrac distance was found to be lower in the litter group than in the cage group at all examined gut sites ($P < 0.001$; Fig. 6A), indicating that litter contact increases the uniformity of microbial species in both the upper and lower intestines as well as in the feces. Nevertheless, while the average weighted UniFrac distance in the litter group was consistently lower than that in the cage group in the duodenum, the distance was higher in the litter group in the jejunum, ileum, and ceca ($P < 0.05$; Fig. 6B). This means that after increasing the microbial species uniformity, the variation in microbial abundance was increased. Since the environment and diet were well controlled, the major factor might be the host’s genetics, which might lead to increased microbial abundance variation [32]. We examined the interindividual UniFrac distance based on the genetic relationship and found that the distance among half sibs was lower than that among unrelated individuals regardless of whether only the microbial species were considered (Fig. 6C) or if the microbial abundance was taken into account (Fig. 6D). Moreover, such a trend was observed in the litter group but not in the cage group, which supported that the increased microbial species uniformity and the host genetic heterogeneity might contribute to the
increased microbial abundance variation. The results suggest that litter contact largely contributes to the uniformity of microbial species, but the host genetic effect on the gut microecology might lead to increased heterogeneity of the microecological impact on the presence of Campylobacter.

Discussion
In this study, we investigated the impact of the gut microecology on Campylobacter presence by comparing the gut microbiota of chickens raised on the litter floor from 0 to 57 days of age with the gut microbiota from chickens raised in individual cages. The results revealed that
the presence of Campylobacter in the chicken gut might (a) benefit from microecological competition among Lactobacillus, Helicobacter, and genera that are halotolerant and aerobic or facultative anaerobic in the upper intestine; (b) be promoted by the increased abundance of obligate anaerobic fermentation microbes in the gut, especially members within the orders Clostridiales and Bacteroidales; (c) occur immensely during 7–28 days of age; and (d) be influenced by host genetics through the impact on the gut microecological composition.

The use of individually caged birds as the comparison group facilitated a better observation of the impact of litter contact on the gut microecology and its association with the presence of Campylobacter in the conventional production environment within a relatively natural habitat setting. Moreover, this contrast facilitated a better understanding of the different contributions of litter contact and host genetics to the gut microecological structure. The result in this study that caged birds were observed to have a significantly lower Campylobacter abundance is consistent with that in the previous study [8]. Compared to the previous study [8], the use of high-throughput sequencing technology and examination of the different gut sites instead of only the feces provided an opportunity to observe the gut microecology underlying the difference in Campylobacter abundance between the two groups.

The microecology in the upper intestine has rarely been reported in previous studies that investigated Campylobacter colonization in chickens. In the current study, the presence of Campylobacter was linked to many obligate anaerobes within the orders Clostridiales and Bacteroidales in the ileum or ceca, which agrees with the observations in previous studies [19, 21, 35]. Moreover, this study found that the Campylobacter presence showed more positive associations with these obligate anaerobic fermentation microbes in the small intestine than with those in the ceca. This might be because Campylobacter is always mucosally adherent in the ceca [33], and its proliferation is likely to be limited by the mucosal surface area, which leads to less linear relationships with these anaerobes in the ceca, although Campylobacter might benefit from anaerobic fermentation by consuming SCFAs [33].

In line with the findings in this study, Lactobacillus has been previously reported to have an inhibitory effect on Campylobacter [36, 37]. Therefore, many Lactobacillus-related probiotic products have been proposed to prevent or reduce the prevalence of Campylobacter [36, 37]. Nevertheless, the results of the current study found that Lactobacillus might not thoroughly clear Campylobacter. Moreover, redundant Lactobacillus might suppress most other microorganisms, including some beneficial microorganisms (Supplementary Table 3), and subsequently reduce the diversity of the gut microbial community and disrupt the microecological balance (Supplementary Fig. 6), which agrees with the previous observation [38]. Thus, although Lactobacillus is effective in controlling Campylobacter, it should be added with caution.

The increased abundance of genera that are halotolerant and aerobic or facultative anaerobic in the small intestine may play an important role in promoting Campylobacter colonization, which was also rarely reported in previous studies. Most of these microbes are abundant taxa in the litter microflora as well (Supplementary Table 4), which agrees with previous observations [10, 39]. These microbes showed negative associations with Lactobacillus (Supplementary Table 3). This might facilitate the colonization of microaerophilic and acid-tolerant Helicobacter in duodenum [10, 39], as Helicobacter showed negative relationships with Lactobacillus and nonlinear relationships with genera that are halotolerant and aerobic or facultative anaerobic (Supplementary Table 4 and Supplementary Fig. 5). Therefore, competitions among these microbes might mitigate the disruption of Lactobacillus to Campylobacter in the chicken gut.

Positive detection of Campylobacter in the gut of newly hatched chicks supports vertical or pseudo-vertical transmission. Some previous studies have reported the negative detection of Campylobacter during the first 1 or 2 weeks post-hatching [15, 40], known as the lag phase [15, 41]. However, Campylobacter was detected in this study in intestinal and fecal samples at 57 days of age where the Campylobacter abundance was $\geq 0$ (A), $\geq 0$ and $\leq 0.5\%$ (B), and $\geq 0$ and $\leq 0.05\%$ (C) and $\leq 0.5\%$ (D). The nonlinear relationship between Helicobacter and Corynebacterium_1 in the duodenum.
Fig. 6 UniFrac distance based microbial community dissimilarity in different groups. Unweighted (A) and weighted (B) UniFrac distances within the litter or cage group at different gut sites. Unweighted (C) and weighted (D) UniFrac distances within half sibs or unrelated individuals at different gut sites. One-way ANOVA was used to examine the significant difference between groups. *** P < 0.001, ** P < 0.01, * P < 0.05, and ns means no significance.
samples from posthatching chicks that originated from a Campylobacter-positive parental population. In line with this finding, Campylobacter has been shown to penetrate eggshells [26] and egg contents through oviduct colonization and fecal contamination [26]. Similarly, some studies have detected Campylobacter in the gut of embryos [42], newly hatched chicks and hatchery fluff [43]. Therefore, the development of Campylobacter colonization might commence at or before hatching, but Campylobacter could hardly colonize the chicks' gut in the first week of life and may immensely colonize from 7 to 28 days of age, which is similar to the results reported in previous studies [15, 35, 44]. This suggests that strategies to prevent vertical or pseudo-vertical transmission should be given attention in farm production.

There are several limitations to the present study. First, the use of solely the V4 region of the 16S rRNA gene in this study might limit the identification of taxonomy at the species level compared to the joint use of regions, such as V3-V5 and V6-V9 [45]. Moreover, a study showed that the use of V2-V3 regions demonstrated higher resolution in taxonomy identification at the genus and species levels than the use of the V3-V4 regions [46]. These results suggest that some species might not be distinguished because of identification limitations in taxonomy at the species level using only the V4 region. However, while some subregion combinations may perform better than others, different subregions would have bias in identifying bacterial taxa [45]. As such, to better understand the taxonomy at a lower-rank level, such as the species or even the strain level, the use of full V1–V9 regions or metagenomics might be more appropriate. Second, although we examined chicks' microbiota from as early as the hatch day and sampled at seven time points, we might miss some information regarding the alteration of gut microbiota as there are gaps between two time points. Therefore, a longitudinal day to day examination might be required in future studies that could help to reveal more accurate changes in the gut microbiota [35]. Moreover, environmental pressure has been found to have a significant impact on the microbial community structure significantly and has been linked to Campylobacter presence [20]; therefore, factors such as stocking intensity should be considered in future studies when investigating the gut microflora and Campylobacter colonization in the chicken gut.

Methods

Subjects, housing, and sample collection

The complete procedure was performed according to the guidelines established by the Animal Care and Use Committee of China Agricultural University (Permit Number: AW08059102–1).

A pure line of slow-growing yellow broilers was used in this study. The birds were obtained from Jiangsu Lihua Poultry Breeding Co., Ltd. in Jiangsu Province, China. A pure line of broilers with Campylobacter detection positivity and clearly recorded kinship was selected. Twelve families were established as the parent generation, and each family consisted of one male and nine females. The birds were kept in individual cages, and artificial insemination was performed to maintain the mating balance and efficiency. At 36 weeks of age, the fertile eggs from the parent generation were collected and incubated. The incubation was performed under the standard incubation procedure, including a strict incubation period, temperature, humidity, and sterilization. After hatching, three full-sib male chicks from each mother were selected and randomly allocated into three groups. One group was used for ileal and cecal sampling, and the other two were retained for the subsequent experiment. One of the retained groups was conventionally raised in the same pen on the floor covered with fresh rice hulls as the litter, and the other group was kept in individual cages to avoid litter contact and cross contamination and served as the comparison group. The bottom of the cage was mesh to allow the feces to drop through, which avoided fecal contact. Both groups were fed with the same diet. To reduce the influences of other environmental factors, the housing conditions were similarly maintained in the two groups according to the housing standards. Since drugs, prebiotics, probiotics, and antibiotics may intensively affect the composition of the gut microbiota [47–49], none of them were used during the experimental period.

Fecal samples were collected from the parents when completing the collection of fertile eggs. We collected meconium from the retained groups and the ileal and cecal mucosal surfaces from the other group after hatching. Fecal samples were collected once the excreta were discharged at 2, 7, 14, 28, 42, and 57 days of age (Supplementary Table 1). The middle of the feces was collected to avoid environmental contamination. Ten families were randomly selected, and three full-sib pairs in each family were randomly selected for the following intestinal sampling. At 57 days of age, these birds were humanely euthanized by cervical dislocation and subsequently dissected. The contents and mucosal surfaces of the duodenum, jejunum, ileum, and ceca were collected immediately after dissection. To ensure the uniformity of samples among individuals, a 10-cm-long fixed section of the duodenum and jejunum, the whole ileum and a pair of ceca were selected for sampling. Therefore, samples from 30 chickens in each group were used for further analysis. In total, there were 330 samples from each group.
(Supplementary Table 1). The contents and mucosa were mixed well before collection. All samples were immediately frozen in liquid nitrogen and then stored at −80°C. Both the intestinal contents and mucosa were sampled, since the microbes from both sources may contribute to host interactions with respect to nutrient metabolism and immunity [50]. Fresh litter samples were collected when the litter was put into the experimental pen. Litter and trough water samples were also collected. Feed samples were collected at the beginning and end of the experiment.

**DNA extraction and 16S rRNA gene sequencing**

Total DNA was extracted from intestinal and fecal samples using the OMEGA E.Z.N.A. Stool DNA Kit (#D4015) following the manufacturer’s instructions. The V4 region (515F-806R) of the 16S rRNA gene was employed to generate indexed libraries for sequencing. All PCRs were carried out in 30μL reactions with 15μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2μM forward and reverse primers, and approximately 10ng template DNA. Thermal cycling consisted of an initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10s, annealing at 50°C for 30s, and elongation at 72°C for 30s. Finally, 72°C for 5 min. Mix the same volume of 1× loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. PCR products were mixed in equidensity ratios. Then, mixture PCR products were purified with GeneJETTM Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) following manufacturer’s recommendations. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific). At last, the library was sequenced on an Ion S5TM XL platform and 400bp single-end reads were generated. Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence.

**Analysis of sequencing data**

The sequence data were resolved to amplicon sequence variants (ASVs) instead of operational taxonomic units (OTUs). ASVs are considered to be a replacement for OTUs based on accumulated evidence and views, such as the improvement in accuracy, reusability, reproducibility and comprehensiveness [51–56]. Sequence quality control, feature table construction and taxonomic annotation were performed using DADA2 [51]. Specifically, sequences were filtered and trimmed to obtain high-quality data. The length of sequences to be trimmed was set as 234bp to ensure that at least 9900 bases could be randomly sampled for quality evaluation at all retained positions. The median of the quality score was 28, and more than 75% of the bases were over 20 at position 234bp. None of the bases was trimmed at the beginning position of the reads, as the quality score of more than 90% of bases at the first 15bp positions was over 20. The parameters HOMOPOLYMER_GAP_PENALTY and BAND_SIZE were set as −1 and 32, respectively, following the recommendation of the tutorial (https://benjjneb.github.io/dada2/faq.html). The singleton ASVs were discarded before removing chimeras because they were generated mainly by sequencing errors. The chimeras were removed using the “consensus” method, and the taxonomy was assigned with the SILVA132 database [57, 58]. Next, microbial diversity analysis was performed using QIIME [59] with a QIME2 pipeline (https://qiime2.org). The results from DADA2 were transformed to the format required in QIME2 and we rarefied the data to 0.9 × lowest numbers of sequences to control for sampling effort in diversity analysis.

The microbial community similarities among samples from different days of age or samples from different gut sites were compared by performing principal coordinates analysis (PCoA) with UniFrac distance. The results were tested for significance by PERMANOVA using vegan in R. The microbial community similarities within the litter or cage group were calculated with interindividual UniFrac distances. Similarly, the microbial community similarities were calculated for individuals with or without genetic relationships. One-way ANOVA was used to examine the significant differences in the UniFrac distance between two groups. The Shannon diversity index was calculated to examine the alpha diversity of the gut microbial community using vegan in R. We performed one-way ANOVA to compare the Shannon diversity index at each gut site between the two groups.

The *Campylobacter* abundances in samples at different days of age and different gut sites are shown with boxplots using ggplot2 in R. The average abundances of abundant phyla in the litter and cage groups at different days of age or different gut sites are shown with alluvial diagrams using geom_alluvium in R. The nonparametric factorial Kruskal-Wallis sum-rank test and linear discriminant analysis were performed in LEfSe [60] to identify the differentially abundant genera between the two groups. To decrease the data noise, only genera with an average relative abundance >0.001 at each sampling site were used for LEfSe.

The associations among microorganisms were determined at the genus level with Pearson correlation analysis by psych in R at each sampling site. Nonlinear association fitting between *Helicobacter* and genera that
are halotolerant and aerobic or facultative anaerobic was performed using geom_smooth in R with the loess method. Only the genera present in over 6 samples were used in the association analysis.

**Measurement of short-chain fatty acids**
A 0.5 g cecal sample was weighed into a 10 ml polypropylene tube, and 8 ml deionized water was added. After using an ultrasonic bath for 30 min, the mixture was centrifuged for 10 min at 8000 rpm. The resulting suspension was diluted 10 times and filtered through a 0.22 μm filter. Then, 25 μL of extracted sample solution was analysed by high performance ion chromatography with ICS-3000 (Dionex, USA) and determined by conductivity detection. The organic acids were separated on an AS11 analytical column (250 mm × 4 mm) and an AG11 guard column under the following gradient conditions: the gradient was carried out with potassium hydroxide; 0–5 min, 0.8–1.5 mM; 5–10 min, 1.5–2.5 mM, and 10–15 min, 2.5 mM, and the flow rate was 1.0 ml/min. One-way ANOVA was performed to test if the SCFA was significantly different between two groups.

**Quantitative real-time PCR**
Numbers of *Campylobacter* spp. (e.g. *C. jejuni*, *C. coli*, *C. lari*, and *C. hyointestinalis*) were quantified by real-time quantitative PCR (qPCR) using specific primers (forward primer: 5′- CACGTGCTACAATGGGATATACAA-3′; reverse primer: 5′- CCGAACTGGGACATATTATGATT-3′), generically targeting *Campylobacter* spp. 16S rDNA sequence according to de Boer et al. [61]. The reference strain was synthesized according to methods as previously described [62], and the 16S rRNA gene was cloned into a pMD-18 T Vector System. Real-time PCR was performed on an Applied Biosystems 7500 thermal cycler using Applied Biosystems® Power SYBR® Green PCR Master Mix. The real-time PCR assay was carried out in a 15 μl volume and contained 1 μl DNA, 7.5 μl Applied Biosystems® Power SYBR® Green PCR Master Mix, 0.3 μl (10 pmol) of forward and reverse primers and 5.9 μl DNase-free water. The cycling conditions consisted of 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 40 s at 72 °C. The standards of *Campylobacter* spp. were diluted to yield a series of 10-fold concentrations and then used for standard curves. The number of copies of *Campylobacter* spp. was transformed by logarithm. The transformed data was used for a one-way ANOVA test to examine the significant difference between two groups.

**Supplementary Information**
The online version contains supplementary material available at https://doi.org/10.1186/s12866-021-02353-5.

**Additional file 1: Supplementary Figure 1.** Average relative abundances of predominant genera in the litter and cage groups at different days of age. Only genera with average relative abundance over 2% are shown with taxonomic annotation. **Supplementary Figure 2.** Average relative abundances of predominant genera in the litter and cage groups in different gut sites at 57 days of age. Only genera with relative abundance over 2% are shown with taxonomic annotations. **Supplementary Figure 3.** Associated with Fig. 4. Heatmap of correlations between *Campylobacter* and microbes in different intestinal segments. Only significant correlations (p < 0.05) over 0.3 or below — 0.25 are shown. **Supplementary Figure 4.** Nonlinear relationships between *Campylobacter* and genera that are halotolerant and aerobic or facultative anaerobic in the small intestine. **Supplementary Figure 5.** Nonlinear relationships between *Helicobacter* and genera that are halotolerant and aerobic or facultative anaerobic in duodenum. **Supplementary Figure 6.** The Shannon index between the litter and cage groups in different gut sites at 57 days of age.

**Additional file 2: Supplementary Table 1.** The chicken genetic family structure and samples of the progeny population. **Supplementary Table 2.** Summary of microbes associated with *Campylobacter*. **Supplementary Table 3.** Correlations between Lactobacillus and microbes at the genus level in different intestinal segments. **Supplementary Table 4.** The relative abundance of microorganisms in used litter.
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