Microbiota from Specific Pathogen-Free Mice Reduces *Campylobacter jejuni* Chicken Colonization

Ayidh Almansour 1,2, Ying Fu 1,2, Tahir Alenezi 1,2, Mohit Bansal 1, Bilal Alrubaye 1,2, Hong Wang 1 and Xiaolun Sun 1,2,*

1 Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA; ama056@uark.edu (A.A.); yingfu@uark.edu (Y.F.); tjalenez@uark.edu (T.A.); mb043@uark.edu (M.B.); bilal.alik8@gmail.com (B.A.); hw001@uark.edu (H.W.)  2 Cell and Molecular Biology (CEMB), University of Arkansas, Fayetteville, AR 72701, USA  * Correspondence: xiaoluns@uark.edu; Tel.: +1-479-575-2322

Abstract: *Campylobacter jejuni*, a prevalent foodborne bacterial pathogen, is mainly transmitted from poultry with few effective prevention approaches. In this study, we aimed to investigate the role of microbiota on *C. jejuni* chicken colonization. Microbiota from specific pathogen-free (SPF) mouse stools were collected as SPF-Aerobe and SPF-Anaerobe. Birds were colonized with SPF-Aerobe or SPF-Anaerobe at day 0 and infected with *C. jejuni* AR101 at day 12. Notably, *C. jejuni* AR101 colonized at 5.3 and 5.6 log10 *C. jejuni* CFU/g chicken cecal digesta at days 21 and 28, respectively, while both SPF-Aerobe and SPF-Anaerobe microbiota reduced pathogen colonization. Notably, SPF-Aerobe and SPF-Anaerobe increased cecal phylum *Bacteroidetes* and reduced phylum *Firmicutes* compared to those in the nontransplanted birds. Interestingly, microbiota from noninfected chickens, SPF-Aerobe, or SPF-Anaerobe inhibited AR101 in vitro growth, whereas microbiota from infected birds alone failed to reduce pathogen growth. The bacterium *Enterobacteriaceae* isolated from infected birds transplanted with SPF-Aerobe inhibited AR101 in vitro growth and reduced pathogen gut colonization in chickens. Together, SPF mouse microbiota was able to colonize chicken gut and reduce *C. jejuni* chicken colonization. The findings may help the development of effective strategies to reduce *C. jejuni* chicken contamination and campylobacteriosis.

Keywords: microbiota transplantation; foodborne pathogen; intestine; bacterial colonization; specific pathogen-free

1. Introduction

*Campylobacter jejuni* colonizes asymptptomatically in the intestinal tract of poultry and causes a prevalent foodborne campylobacteriosis around the world [1,2]. *C. jejuni* resistant to macrolides, fluoroquinolones, aminoglycosides, or carbapenems has been detected in samples from children and adults worldwide [1–7]. More than 20 cases of campylobacteriosis per 100,000 population were reported in the USA in 2019 [8], and more than 220,000 people were affected in Europe in 2019 [9]. The case number was more than the total incidences induced by eight other bacterial pathogens [10]. More than 14.35 cases per 0.1 million people were caused by the pathogen in 2020 [11]. Moreover, *C. jejuni* often causes severe post-infectious complications, such as arthritis [12], the neurodegenerative disorder Guillain–Barré syndrome [13], irritable bowel syndrome [14], and inflammatory bowel diseases (IBD) [15,16].

To reduce campylobacteriosis, different measures have been implemented to reduce enteritis by reducing *C. jejuni* contamination in animal food, particularly pre- and post-harvest poultry. The intervention methods include strict biosecurity on farms [17], vaccines [18], probiotics [19], phages [20], decontamination of poultry carcasses in the post-slaughter process [21], facility design and management, reducing contamination in feed, transportation, and other sources, and other strategies [2]. It is estimated that decreasing *Campylobacter*...
count on chicken carcasses by 100 times decreases human campylobacteriosis 30-fold [22]. Although those reduction measurements reduce some C. jejuni contamination, improved and alternative strategies are much needed, as reflected in the consistent high level of campylobacteriosis incidence reported in the Morbidity and Mortality Weekly Report from the Infectious Disease Database at CDC between 1996 and 2017 [23].

The gastrointestinal tract of humans and animals is inhabited by trillions of microbes, collectively called the microbiota [24,25]. The gut microbiota modulates essential host physiology and various host functions such as the intestinal barrier, nutrition, and immune homeostasis [25–29]. Specific pathogen-free (SPF) Il10−/− mice are naturally resistant to C. jejuni 81–176-induced colitis, while the mice become susceptible to campylobacteriosis after being treated with the broad-spectrum antibiotic clindamycin [30]. Sequencing and bioinformatic analysis of 16S rDNA revealed that microbiota-mediated bile acid metabolism was essential for preventing C. jejuni-induced colitis. Increasing evidence is emerging on gut microbiota preventing C. jejuni colonization in poultry [31–33]. Apart from naturally transmitting microbiota from wild hens to turkey chicks, the turkey microbiota transmission is disrupted in modern industrialized poultry production, partly because eggs are hatched by a hatchery instead of hens [34]. Poultry chicks obtain their microbiota from the environment and/or farms, where most of the microbes are not natural inhabitants of the bird gut [35]. The application of antibiotics as growth promoters further drives the dysbiosis of birds in commercial poultry production [36]. In our previous studies, we found that transplanting bile acid deoxycholic acid-modulated microbiota to hatched chicks reduced the colonization of C. jejuni human clinical isolate 81–176 and chicken isolate AR101 in pre-harvest chickens [37]. Because SPF mice are naturally resistant against a C. jejuni infection [30,38], in this study, we hypothesized that SPF mouse microbiota would be able to colonize chickens and reduce C. jejuni chicken colonization. Our data indicate that the mouse SPF-Aerobe and SPF-Anaerobe microbiota shaped the chicken intestinal microbiota. Furthermore, the SPF-Aerobe and SPF-Anaerobe indeed reduced C. jejuni AR101 in vitro growth and chicken colonization. These findings will help the development of effective strategies against C. jejuni chicken colonization.

2. Results

2.1. Mouse Microbiota Reduced C. jejuni AR101 Chicken Colonization

Mouse SPF-Aerobe and SPF-Anaerobe microbiota was prepared from SPF mouse stools and transplanted to zero-day-old chicks. DNA from C. jejuni chicken isolate AR101 was isolated, and 16S rDNA was PCR-amplified, Sanger-sequenced, and confirmed to be in 99.0% alignment with C. jejuni. The birds were infected with AR101 at day 12. Consistently with our previous reports [37], C. jejuni was not detected in noninfected birds, suggesting clean housing at our poultry facility. Notably, mouse SPF-Aerobe and SPF-Anaerobe microbiota reduced C. jejuni AR101 cecal colonization by more than 1-log compared to that of only infected birds (Cj AR101) at day 21 (3.8 ± 0.2 and 4.1 ± 0.0 vs. 5.3 ± 0.4 log10 C. jejuni CFU/g cecal digesta, respectively) (Figure 1A). The SPF-Aerobe and SPF-Anaerobe continued to reduce C. jejuni chicken colonization compared to that of the infected control birds at day 28 (4.0 ± 0.6 and 4.9 ± 0.1 vs. 5.6 ± 0.2 log10 C. jejuni CFU/g cecal digesta, respectively) (Figure 1B). Notably, the SPF-Anaerobe microbiota with or without C. jejuni AR101 infection increased the accumulative body weight compared to that of noninfected birds from day 0 to day 28 (1606 ± 17.7 and 1683 ± 43.1 vs. 1463 ± 47.6 g/bird, respectively) (Figure S1), while SPF-Aerobe microbiota did not increase the bird weight gain. These results suggest that SPF-Aerobe and SPF-Anaerobe effectively reduce C. jejuni AR101 colonization in chickens.
Figure 1. Murine microbiota reduced \textit{C. jejuni} AR101 chicken colonization. Zero-day-old broiler chickens precolonized with SPF-Aerobe and SPF-Anaerobe were infected with \textit{C. jejuni} AR101 at 12 days of age. The birds were euthanized at days 21 and 28. The bird cecal digesta was collected, serially diluted, and cultured on \textit{Campylobacter}-selective agar plates prepared in-house at 42 °C under microaerobic atmosphere. (A) \textit{C. jejuni} chicken colonization in the ceca of the birds at day 21. The bird number for each group was: noninfected (n = 10), Cj AR101 (n = 10), SPF-Aerobe (n = 5), and SPF-Anaerobe (n = 10). (B) \textit{C. jejuni} chicken colonization in the ceca of the birds at day 28. The bird number for each group was: noninfected (n = 20), Cj AR101 (n = 20), SPF-Aerobe (n = 10), and SPF-Anaerobe (n = 20). All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

2.2. SPF-Aerobe and SPF-Anaerobe Modulated the Chicken Microbiota

We reasoned that the colonization reduction of AR101 in chickens might come from chicken microbiota alteration by the mouse microbiota transplantation. To assess this hypothesis, we used phylum-specific primers to analyze the microbiota composition change. Notably, SPF-Aerobe and SPF-Anaerobe reduced the relative abundance of the phylum \textit{Firmicutes} compared to that of uninfected birds (61.3 and 52.9 vs. 97.5%) and infected birds (51.9 and 50.9 vs. 86.7%, respectively), while the relative abundance of \textit{Bacteroidetes} was increased compared to that of uninfected birds (38.4 and 44.7 vs. 2.3%) and infected birds (42.0 and 47.6 vs. 12.4%, respectively) (Figure 2). Interestingly, \textit{C. jejuni} colonization modulated chicken cecal microbiota of the phyla \textit{Bacteroidetes} and \textit{Firmicutes}. Importantly, most of the relative abundance was significant (Table 1). These results indicate that SPF-Aerobe, SPF-Anaerobe, and \textit{C. jejuni} were able to colonize and change the microbiota in the chicken gut.

2.3. Chicken Noninfected Microbiota and Mouse SPF Microbiota Reduced \textit{C. jejuni} Growth

Upon validation of transplanted mouse SPF microbiota reducing \textit{C. jejuni} chicken colonization, we reasoned that the mouse SPF microbiota would directly inhibit \textit{C. jejuni} AR101 growth, while the chicken microbiota would not. To examine this hypothesis, \textit{C. jejuni} AR101 inoculum was co-cultured with microbiota from noninfected, SPF-Aerobe, and SPF-Anaerobe chickens in the \textit{Campylobacter} Enrichment (CE) Broth at 42 °C for 24 h under anaerobic conditions. Notably, both SPF-Aerobe and SPF-Anaerobe reduced \textit{C. jejuni} AR101 by more than 1-log compared to the \textit{C. jejuni} AR101 culture-alone group (6.8 ± 0.2 and 6.3 ± 0.1 vs. 8.6 ± 0.3 $\log_{10}$ \textit{C. jejuni} CFU/mL, respectively) (Figure 3). Interestingly, the microbiota from noninfected chickens also reduced \textit{C. jejuni} AR101 growth by more than 2-log compared to that in the \textit{C. jejuni} AR101 culture alone (6.4 ± 0.4 vs. 8.6 ± 0.3 $\log_{10}$ \textit{C. jejuni} CFU/mL, respectively). Notably, each microbiota at 24 h increased the number of CFU compared to that at 0 h (Figure S2).
Table 1. Significant p-values of relative abundance at the phylum level between groups.

| Group A                          | Compared to Group B | Phylum       | p-Value |
|----------------------------------|---------------------|--------------|---------|
| Noninfected                      | SPF-Aerobe          | Bacteroidetes| <0.001  |
|                                  | SPF-Aerobe          | Firmicutes   | <0.001  |
|                                  | SPF-Anaerobe        | Bacteroidetes| <0.001  |
|                                  | SPF-Anaerobe        | Firmicutes   | <0.001  |
|                                  | Cj AR101            | Bacteroidetes| 0.02    |
|                                  | Cj AR101            | Firmicutes   | 0.04    |
| Cj AR101                         | SPF-Aerobe + Cj AR101| Bacteroidetes| <0.001  |
|                                  | SPF-Aerobe + Cj AR101| Firmicutes   | <0.001  |
|                                  | SPF-Anaerobe + Cj AR101| Bacteroidetes| <0.001  |
|                                  | SPF-Anaerobe + Cj AR101| Firmicutes   | <0.001  |

Figure 2. SPF microbiota modified the chicken microbiota at day 28. The birds were colonized with microbiota and infected with C. jejuni AR101 at day 12 as in Figure 1. Cecal digesta was collected at day 28, and DNA was extracted. Real-time PCR was performed to calculate bacterial composition at the phylum level. The detailed p-values were listed in Table 1. The results are representative of three independent experiments.

Figure 3. In vitro co-culture of noninfected chicken microbiota and C. jejuni AR101. AR101 was co-cultured for 24 h with microbiota from noninfected, SPF-Aerobe, or SPF-Anaerobe birds in vitro. AR101 growth was quantified by serially diluting and plating on the Campylobacter-selective agar plates. All graphs depict the mean + SEM. Significant if p < 0.05. The results are representative of three independent experiments.
Because of the unexpected result of noninfected chicken microbiota reducing C. jejuni AR 101 in vitro growth, we then modulated our hypothesis that C. jejuni possibly modulated chicken microbiota for its growth and colonization. To address this reasoning, we co-cultured C. jejuni AR101 with chicken microbiota from only infected birds (Cj-MB), transplanted with SPF-Aerobe and infected birds (Cj-SPF-Aerobe), and transplanted with SPF-Anaerobe and infected birds (Cj-SPF-Anaerobe). Interestingly, the three-chicken microbiota themselves could not grow a single colony on the Campylobacter-selective plates (Figure 4), suggesting that C. jejuni lost culturability after storing with microbiota. Notably, Cj-MB did not reduce C. jejuni AR101 in vitro growth compared to that in positive control of Cj AR101 culture alone. Consistently, Cj-SPF-Aerobe and Cj-SPF-Anaerobe reduced C. jejuni AR101 in vitro growth by more than 3-log compared to that in the Cj AR101 culture-alone group (3.7 ± 0.6 and 0.8 ± 0.5 vs. 7.3 ± 0.1 log10 C. jejuni CFU/mL, respectively). Consistently, each microbiota at 24 h increased number of CFU compared to that at 0 h (Figure S3). These results suggest that C. jejuni modulated the chicken microbiota for its growth and colonization, while the transplanted mouse SPF microbiota resisted against pathogen growth.

Figure 4. In vitro co-culture of C. jejuni-modulated microbiota and C. jejuni AR101. C. jejuni AR101 was co-cultured with microbiota from infected-alone birds (Cj-MB), transplanted with SPF-Aerobe and infected birds (Cj-SPF-Aerobe), and transplanted with SPF-Anaerobe and infected birds (Cj-SPF-Anaerobe). AR101 growth was quantified by serially diluting and plating on the Campylobacter-selective agar plates. All graphs depict the mean + SEM. Significant if $p < 0.05$. The results are representative of three independent experiments.

2.4. An Aerobic Bacterial Isolate Reduced C. jejuni AR101 In Vitro Growth

Next, we wanted to identify and isolate the individual bacteria from the protective SPF microbiota. About 100 bacterial colonies were isolated using BHI plates at 42 °C under anaerobic conditions for 48 h. The colonies were individually co-cultured with C. jejuni for 24 h, and then C. jejuni was enumerated on the Campylobacter-selective plates prepared in-house. Unfortunately, none of the bacteria were able to inhibit C. jejuni growth using the co-culture method. By accident, during one chicken trial, a bacterial colony from birds gavaged with mouse SPF-Aerobe was able to grow with pink color on the Campylobacter-selective plate compared to the dark red color of C. jejuni. The bacterial colony was selected and later named Enterobacter102. We reasoned that this bacterium might resist C. jejuni infection. Enterobacter102 was rod-shaped, stained Gram-negative, and had the same size as E. coli. Enterobacter102 also grew in pink colonies on a MacConkey agar plate. The DNA from Enterobacter102 was isolated, and 16S rDNA was PCR-amplified, Sanger-sequenced, and confirmed to be in 95% alignment with Enterobacter sp. To functionally
dissect the interaction between Enterobacter102 and C. jejuni AR101, in vitro co-culture was performed. Interestingly, Enterobacter102 showed the ability to reduce C. jejuni AR101 colonization by more that 2-log compared to that in the Cj AR101 culture-alone group (4.6 ± 0.1 vs. 7.3 ± 0.1 log10 C. jejuni CFU/mL) (Figure 5). Not surprisingly, the number of Enterobacter102 increased at 24 h compared to that at 0 h (Figure S4). Although the reduced C. jejuni could result from depleted nutrients in the presence of a microbiota (SPF microbiota or Enterobacter102), the comparable growth between Cj AR101 and Cj-MB AR101 in Figure 4 suggested that the microbiota was an important factor influencing C. jejuni growth. These results suggested that Enterobacter102 has potential to reduce C. jejuni AR101 chicken colonization.

![Figure 5](image)

**Figure 5.** In vitro co-culture of Enterobacter102 and C. jejuni AR101. C. jejuni AR101 was co-cultured with Enterobacter102. AR101 growth was quantified by serially diluting and plating on Campylobacter-selective agar plates. All graphs depict the mean + SEM. Significant if p < 0.05. The results are representative of three independent experiments.

2.5. Enterobacter102 Reduced C. jejuni AR101 Chicken Colonization

Encouraged by the result of Enterobacter102 reducing C. jejuni AR101 in vitro growth, we then performed chicken experiments. The birds were colonized with 10⁸ CFU/chick of Enterobacter102 at day 0, infected with C. jejuni at day 12, and euthanized at days 21 and 28. Consistently with the in vitro experiments, Enterobacter102 reduced C. jejuni AR101 chicken colonization by more than 1-log at day 21 in comparison to that in only infected birds of the Cj AR101 group (4.0 ± 0.0 vs. 5.3 ± 0.4 log10 C. jejuni CFU/g cecal digesta) (Figure 6A). Notably, Enterobacter102 continued to reduce C. jejuni AR101 chicken colonization by more than 2-log at day 28 (2.4 ± 0.9 vs. 5.7 ± 0.2 log10 C. jejuni CFU/g cecal digesta) (Figure 6B). These results suggest that the bacterial isolate of Enterobacter102 inhibited C. jejuni AR101 growth and reduced the pathogen’s chicken colonization.
A notable observation from this study is that the mouse microbiota was able to be successfully transplanted into chickens and to reduce C. jejuni chicken colonization. It is a well-known medical practice to transplant a healthy donor’s microbiota to treat a human Clostridium perfringens infection [40]. The microbiota compositions of human recipients are comparable to those of the human donor’s, and the C. difficile infection is reduced. Consistently, microbiota composition in recipient piglets is similar to that of human donors in an intermammalian microbiota transplantation [41], suggesting that it is feasible to transplant microbiota between animals within the class level of Mammalia. In the current study, we successfully transplanted mouse (class Mammalia) microbiota to chickens (class Aves), suggesting it is possible to transplant microbiota between animals within the phylum level of Chordata. Apparently, the difference of body temperature (42 °C in chickens and 37 °C in mice) and intestinal anatomy between the animals did not
reduce the donor mouse microbiota colonization in the recipient chickens. A meta-data analysis study showed that chicken microbiota at the phylum level is mainly comprised of 13 phyla, including Firmicutes (70.0%), Bacteroidetes (12.3%), Proteobacteria (9.3%), and other small proportions of Actinobacteria, Cyanobacteria, Spirochaetes, Synergistetes, Fusobacteria, Tenericutes, and Verrucomicrobia [42]. Consistent with this finding, we found that birds without a mouse microbiota transplantation had the phylum Firmicutes majority, while microbiota-transplanted birds dramatically reduced Firmicutes and increased Bacteroidetes, independently of C. jejuni infection. Interestingly, the microbiota in mice is composed of the phyla Firmicutes at 54% and Bacteroidetes at 30% [43], which is close to the composition of our transplanted chicken microbiota. A field survey study reported that birds from the farms with the highest Campylobacter counts show the highest percentage of Firmicutes and the lowest percentage of Bacteroidetes in their microbiota, although microbiota composition is highly variable between or within farms [44]. In addition, the significant reduction of C. jejuni colonization by SPF-Aerobe and SPF-Anaerobe microbiota in both days 21 and 28 suggested that the microbiota may continue to reduce pathogen colonization for a longer period of time. This experiment was cut short because of the pen size constrain. It would be interesting in the future to conduct follow-up experiments to reduce C. jejuni colonization by SPF microbiota for birds at the market age of days 35–45. Together, these data showed that mouse SPF microbiota is transplantable to reduce C. jejuni chicken colonization.

After the evaluation of the protective effect of the mouse SPF microbiota, it is imperative to isolate and identify individual bacteria in the microbiota against C. jejuni chicken colonization for further functional evaluation. In a human longevity study, Sato and colleagues have plate-cultured, isolated, and evaluated a group of 68 bile acid metabolizing bacteria [45]. They found that Parabacteroides merdae and Odoribacteraceae strains produced isoalloLCA and reduced Gram-positive multidrug-resistant pathogens, such as C. difficile and vancomycin-resistant Enterococcus faecium [45]. A microbiota with higher level of genera Clostridium XI, Bifidobacterium, and Lactobacillus is associated with resistance to C. jejuni-induced colitis in mice [30]. Interestingly, probiotics Bifidobacterium longum PCB133 and a xylo-oligosaccharide do not decrease C. jejuni chicken colonization [46]. We have co-cultured C. jejuni with various ATCC or lab-isolated bacteria, such as Bifidobacterium longum and Clostridium scindens, and we did not find the bacteria to reduce C. jejuni in vitro growth (data not shown). During our search for individual microbiota against C. jejuni, we found that the Enterobacter102 from microbiota of SPF-Aerobe grew as pink colonies on the Campylobacter-selective plates. Later, we found that Enterobacter102 reduced C. jejuni in vitro growth and chicken colonization. Probiotic application of Enterobacter sp. improves both Mediterranean fruit fly (medfly) pupal and adult productivity and reduces rearing duration [47]. Most other reports showed that Enterobacter sp. is a pathogen and induces intestinal inflammation [48,49]. Future research on how Enterobacter102 reduces C. jejuni growth and chicken colonization is much needed. We are working on identifying Enterobacter102 and other bacterial candidates by culture-isolation and 16S rDNA Sanger sequencing. Together, these data suggest that individual bacteria in the SPF microbiota might be able to be isolated and used to reduce C. jejuni growth and chicken colonization.

Another interesting finding from the current study is that the microbiota from noninfected birds at day 28 was able to reduce C. jejuni in vitro growth, while microbiota from infected-alone birds failed to reduce pathogen growth. The results suggest that C. jejuni might have modulated the chicken microbiota facilitating pathogen colonizing and thriving in the gut. It is a consensus that intestinal microbiota influences C. jejuni colonization and induction of enteritis [24,50], as also discussed in the paragraphs above. However, few reports showed that C. jejuni modulates the microbiota to benefit its own colonization. Salmonella Enteritidis infection reduces the overall diversity of the chicken microbiota population with an expansion of the Enterobacteriaceae family for promoting pathogen colonization [51]. In the current study, we found that a C. jejuni infection increased the phylum Bacteroidetes compared to that in noninfected birds. Future research is needed to identify which specific bacteria are increased to facilitate C. jejuni colonization.
In conclusion, the mouse SPF microbiota was able to colonize chicken ceca and reduced *C. jejuni* chicken colonization. The reduction of *C. jejuni* chicken colonization might come from reduced bacteria in the phylum *Firmicutes* and/or increased bacteria in the phylum *Bacteroidetes*. Notably, *Enterobacter*102 reduced *C. jejuni* in vitro growth and chicken colonization. Altogether, these findings provide a feasible strategy to reduce *C. jejuni* chicken contamination and human campylobacteriosis.

4. Materials and Methods

4.1. Mouse Microbiota Preparation and Chicken Experiments of Microbiota Transplantation and *C. jejuni* Infection

The performed animal experiments were in accordance with the Animal Research: Reporting of In Vivo Experiments (https://www.nc3rs.org.uk/arrive-guidelines accessed on 22 August 2019) and approved by the Institutional Animal Care and Use Committee of the University of Arkansas (protocols No. 20009 for mice and 20111 for chickens). For the bird experiment with SPF microbiota, a total of 135 zero-day-old Cobb 500 broiler chicks were randomly allocated into cohorts of 15–30 birds per group, as detailed in Supplementary Table S1. The birds obtained from Cobb-Vantress (Silloam Springs, AR, USA) were neck-tagged and randomly assigned to floor pens with a controlled age-appropriate environment. The birds were fed a corn-soybean meal-based starter diet during days 0–10 and a grower diet during days 11–28. The basal diet was formulated as described earlier [37,52].

Stool from eight-week-old SPF BL6 *Il10*−/− mice fed a chew diet was freshly collected and immediately suspended in 30% glycerol PBS stock and stored at −80°C. The stool samples were cultured on brain heart infusion (BHI, BD Biosciences, Franklin Lakes, NJ, USA) agar plates at 42°C for 48 h under aerobic or anaerobic conditions using the GasPak system (BD Biosciences, Franklin Lakes, NJ, USA) and collected as SPF-Aerobe and SPF-Anaerobe microbiota. The microbiota was added glycerol at final 30% and stored at −80°C. Before the chicken colonization experiment, the SPF-Aerobe and SPF-Anaerobe microbiota were cultured on a BHI plate for 48 h, collected in PBS, and enumerated by OD600 and plating. OD600 of 1 was estimated at about 10^8 CFU/mL. At chicken experiments, chicks at day 0 were orally gavaged once with PBS or 10^8 CFU/bird SPF-Aerobe or 10^8 CFU/bird SPF-Anaerobe. For the chicken experiment of *Enterobacter*102, a total of 90 zero-day-old Cobb 500 broiler chicks were randomly allocated into cohorts of 30 birds per group. The birds were fed and raised similarly to those in the SPF microbiota experiment. The chickens were orally gavaged once with PBS or 10^8 CFU/bird of *Enterobacter*102 in 0.5 mL/bird on day 0. Two days before infection, frozen stock of *C. jejuni* AR101 (isolated at Dr. Billy Hargis’s lab at University of Arkansas at Fayetteville) were cultured microaerobically at 42°C for 48 h on *C. jejuni*-selective blood plates. The motility of *C. jejuni* was ensured under a microscope as described before [53] and routinely examined on semisolid MH (0.4% agar) plates. The *bacterium* was also serially diluted, cultured on the *Campylobacter*-selective plates, and enumerated 48 h later. The *Campylobacter*-selective plate was prepared in-house and it consisted of Bolton’s Campylobacter Enrichment (CE) Broth (Neogen Food Safety, Lasing, MI, USA), 1.5% agar (VWR, USA, OH), 5% lyzed horse blood (VWR, Radnor Township, PA, USA), five antibiotics (20 mg/L cefoperazone, 50 mg/L cycloheximide, 20 gm/L trimethoprim, 20 mg/L vancomycin, and 0.35 mg/L polymyxin B), 500 mg/L ferrous sulfate, and 200 mg/L triphenyl-tetrazolium chloride (TTC) (all from Sigma-Aldrich, St. Louis, MO, USA). The ferrous sulfate and TTC were used to make *C. jejuni* colonies dark red. The birds were gavaged with 1 mL PBS or 10^9 CFU/bird *C. jejuni* AR101 at day 12 [37]. Chicken body weight was measured at days 0 and 28. Because of the pen size constraints, the birds were randomly euthanized at days 21 and 28 to collect cecal samples for enumerating *C. jejuni*, and the exact bird numbers are listed in figure legends.

This experiment was conducted until 28 days of age because of the pen size constrain. Cecal digesta samples of all the groups were collected for DNA isolation. Another set of cecal digesta were serially diluted ten-fold with sterile PBS and cultured on the *Campylobacter*-selective plates at 42°C for 48 h under a microaerophilic atmosphere. Emerged colonies
were positively determined as C. jejuni only when they were dark red and shining, round, and with a smooth surface. The colonies were also examined under a microscope for size and motility evaluation [53]. The CFU per gram digesta was then calculated.

4.2. Estimation of Microbiota Composition at Phylum Level

Cecal digesta samples were collected, and DNA was extracted using bead beater disruption and phenol: chloroform separation method as described before [54]. Briefly, 0.1 g of fecal sample suspended in 500 µL PBS was transferred to a 2 mL screw cap tube containing 85 µL of 10% SDS solution, 500 µL of phenol/chloroform (25:24), and 0.3 g sterile 0.1 mm zirconia beads (BioSpec, Bartlesville, OK, USA). The samples were homogenized on a Fisherbrand Bead Mill 24 Homogenizer (Fisher Scientific, Pittsburgh, PA, USA) for 3 × 30 s at high speed with a 10 s pause for each run. After centrifugation, the supernatant was further extracted twice with 500 µL of chloroform (25:24), and the top aqueous layer was collected and mixed with 1/10 Vol (~50 µL) 3M sodium acetate (pH 5.2) and 2.5 Vol (~1.25 mL) ethanol overnight at –20°C. After centrifugation, the DNA pellet was washed once with 70% ethanol and resuspended in 100 µL DNase/RNase-free H2O. The abundance levels of five phyla of gut bacteria were determined by real-time PCR according to the manufacturer’s recommendation. Briefly, each PCR reaction mixture comprised 4 µL of BioRad iTaq Universal SYBER Green Super mix (BioRad, Hercules, CA, USA), 1.6 µL of template DNA (~4 ng), 0.6 µL of 5 µM primer mix, and 1.8 µL of DNase/RNase H2O. The amplification reaction was performed in a BioRad 384 Real-Time PCR machine (BioRad, Hercules, CA, USA) using the following program: 1 min at 95°C, followed by 30 cycles of 30 s each at 95°C, 60 s at 60°C. The gene primers [37] used included universal 16S rRNA: 16S5′-CTCCTACGGGAGGCAGCAAA-3′, 16S1392R 5′-ACGGCGGTGTGTRC-3′; α-proteobacteria: α682F 5′-CAATGTCAGAGGTGAAT-3′, 908αR 5′-CCCCGTCAATTCCTTTGAGTT-3′; γ-proteobacteria: 1080γF 5′-TCTGCAGTCGTGTGTYTGA-3′, γ1202R 5′-CTGTAAGGGCCATGATG-3′; Bacteroidetes: 798cBF 5′-CRAAACAGGATTAGATACCCT-3′, eb967R 5′-GGTAAAGTTCTCTCCGGTAT-3′; Firmicutes: 928FirmF 5′-TGAAACTYAAAGGAATTGACG-3′, 1040FirmR 5′-ACCATGCACCACCTGTC-3′, Actinobacteria: Act920F3 5′-TACGGCCGCAAGGCTA-3′, Act1200R 5′-TCTGCAGTCGTGTGTYTGA-3′; Actinobacteria: Act920F3 5′-TACGGCCGCAAGGCTA-3′, Act1200R 5′-TCTGCAGTCGTGTGTYTGA-3′, 16S1392R 5′-TCTGCAGTCGTGTGTYTGA-3′, 5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R: 5′-CTGTCAGCCTCCTACGGGGAGGCAGCAA-3′, Act920F3 5′-TACGGCCGCAAGGCTA-3′, Act1200R 5′-TCTGCAGTCGTGTGTYTGA-3′, 16S1392R 5′-TCTGCAGTCGTGTGTYTGA-3′.

The relative percentage of each phylum was determined by real-time PCR quantification method [55] similar to that in this paper [55]. Briefly, the 2−ΔΔCT value of each phylum gene expression Ct in one sample was calculated using the universal 16S rRNA gene expression Ct. The percentage of each phylum was then calculated by the phylum 2−ΔΔCT value in one sample divided by the sum of all phylum 2−ΔΔCT values in the same sample and multiplied by 100.

4.3. Isolation of Enterobacter102

When chicken cecal digesta were cultured on C. jejuni selective plates, pink colonies were grown on the plate, compared to dark red C. jejuni colonies. The pink colony was named Enterobacter102. Under a light microscope, Enterobacter102 was rod-shaped and larger than C. jejuni. Enterobacter102 was able to grow aerobically, stained Gram-negative, and showed pink colonies on a MacConkey plate.

4.4. Identification of Bacterial Species Using 16s rDNA and Sanger Sequencing

Either C. jejuni AR101 or Enterobacter102 was derived from a single colony. To isolate DNA for Sanger sequencing, the bacteria were spread on the respective agar plates. The bacteria were collected, and DNA was extracted. Genomic DNA from C. jejuni AR101 or Enterobacter102 was amplified by PCR of the 16S rDNA gene region with universal primers (27Fw1: 5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R: 5′-CGGTACCTGTTAGACTT-3′) following the instructions in this webpage https://chmi-sops.github.io/mydoc_16S_Sanger.html (accessed on 20 July 2020). The PCR products were gel-purified and Sanger-sequenced at Eurofins Scientific using primers of 27Fw1, 1492R, and universal primer 515Fw2: 5′-GTGCCAGCMGCGCGGTAA-3′. The sequences were assembled and aligned
using the NCBI genome database. The bacteria were given species names with >98.0% and 95.0% of 16S rDNA sequence homology for Campylobacter jejuni and Enterobacter sp., respectively. The 16s rDNA sequences were uploaded at NCBI with submission numbers of SUB10285129 and SUB10285090.

4.5. In Vitro Co-Culturing of C. jejuni with Various Microbiota

The impact of various microbiota on C. jejuni growth was evaluated. Briefly, C. jejuni AR101 from frozen stocks was cultured and grown on the Campylobacter-selective plates in a microaerophilic atmosphere for 48 h using the GasPak system (BD Biosciences, Franklin Lakes, NJ, USA). C. jejuni at 6.3 × 10⁸ CFU was co-cultured with noninfected microbiota at 2.0 × 10⁹ CFU, SPF-Aerobe at 1.8 × 10⁸ CFU, or SPF-Anaerobe at 8.4 × 10⁸ CFU in 1 mL of CE broth. C. jejuni at 7.7 × 10⁸ CFU was co-cultured with Cj-MB at 6.7 × 10⁷ CFU, Cj-SPF-Aerobe at 1.6 × 10⁸ CFU, or Cj-SPF-Anaerobe at 1.5 × 10⁹ CFU in 1 mL of CE broth. C. jejuni at 1.8 × 10⁶ CFU and 4.4 × 10⁷ CFU Enterobacter102 were co-cultured in 1 mL of CE broth. The experiments were carried out in triplicate. Because C. jejuni growth would be reduced within 24 h in anaerobic conditions [54], the co-culture bacteria were incubated for 24 h at 42 °C under anaerobic conditions using the GasPak system (BD Biosciences, Franklin Lakes, NJ, USA) to mimic cecal air conditions. C. jejuni growth was measured by serial dilution and plating on the Campylobacter-selective plates for enumeration. When co-culture with C. jejuni, Enterobacter102 was counted with pink colonies compared to dark red colonies of C. jejuni. Cj-MB, Cj-SPF-Aerobe, and Cj-SPF-Anaerobe themselves could not grow a single colony on the Campylobacter-selective plates, suggesting that C. jejuni lost culturability after storing with microbiota.

4.6. Statistical Analysis

All values are shown as mean ± standard error of the mean as indicated. Differences between groups were analyzed using the nonparametric Mann–Whitney U test performed using GraphPad Prism 7.0 software. C. jejuni CFU was transformed with a formula of log10 (CFU + 1). The results were considered statistically significant if p-values were <0.05.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10111387/s1, Figure S1. Accumulative body weight gain during days 0–28. Figure S2. SPF microbiota growth co-cultured with C. jejuni for 24 h. Figure S3. Cj-SPF microbiota growth co-cultured with C. jejuni for 24 h. Figure S4. Enterobacter102 growth co-cultured with C. jejuni for 24 h. Table S1. Number of birds in each group of SPF microbiota experiments.

Author Contributions: A.A., Y.F., T.A. and X.S. designed the experiments and wrote the manuscript with input from co-authors M.B., B.A. and H.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grants of the Arkansas Biosciences Institute, Research Incentive grant from UofA System Division of Agriculture, USDA National Institute of Food and Agriculture (NIFA) Hatch project 1012366, NIFA Hatch/Multi State project 1018699, and NIFA SAS 2019-69012-29905 to X. Sun. This research was also supported by grants of NIFA project 2020-67016-31346 to X. Sun. This research was also supported by Poultry Federation Scholarship to A. Almansour, Y. Fu, and M. Bansal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Institutional Review Board Statement: The animal protocols No. 20009 and 20011 were approved by the Institutional Animal Care and Use Committee of the University of Arkansas at Fayetteville.

Data Availability Statement: The data are presented in this paper.

Acknowledgments: The authors would like to thank for the support from the staff at Poultry Health Laboratory and Feed Mill in the Department of Poultry Science at University of Arkansas, Fayetteville.

Conflicts of Interest: The authors declare no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.
References

1. WHO. Campylobacter. Available online: https://www.who.int/news-room/fact-sheets/detail/campylobacter (accessed on 21 October 2021).

2. Hazards, E.P.; Olofsson, K.; Alleva, A.; Alvarenga-Ordonez, A.; Bolten, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Herman, L.; et al. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. EFSA J. 2021, 19, e06651. [CrossRef]

3. Pham, N.T.; Thongprachum, A.; Tran, D.N.; Nishimura, S.; Shimizu-Onda, Y.; Tran, Q.D.; Khamrin, P.; Ukarapol, N.; Kongsricharoen, T.; Kominke-Aizawa, S.; et al. Antibiotic Resistance of Campylobacter jejuni and C. coli Isolated From Children with Diarrhea in Thailand and Japan. Jpn. J. Infect. Dis. 2016, 69, 77–79. [CrossRef] [PubMed]

4. Bae, J.; Oh, E.; Jeon, B. Enhanced transmission of antibiotic resistance in Campylobacter jejuni biofilms by natural transformation. Antimicrob. Agents Chemother. 2014, 58, 7573–7575. [CrossRef] [PubMed]

5. Hou, F.Q.; Sun, X.T.; Wang, G.Q. Clinical manifestations of Campylobacter jejuni infection in adolescents and adults, and change in antibiotic resistance of the pathogen over the past 16 years. Scand. J. Infect. Dis. 2012, 44, 439–443. [CrossRef] [PubMed]

6. Szczepanska, B.; Kaminski, P.; Andriejewska, M.; Spica, D.; Kartanas, E.; Ulrich, W.; Jerzak, L.; Kasprzak, M.; Bochenski, M.; Klawe, J.J. Prevalence, virulence, and antimicrobial resistance of Campylobacter jejuni and Campylobacter coli in white stork Ciconia ciconia in Poland. Foodborne Pathog. Dis. 2015, 12, 24–31. [CrossRef] [PubMed]

7. Zhao, S.; Mukherjee, S.; Chen, Y.; Li, C.; Young, S.; Warren, M.; Abbott, J.; Friedman, S.; Kabera, C.; Karlsson, M.; et al. Novel gentamicin resistance genes in Campylobacter isolated from humans and retail meats in the USA. J. Antimicrob. Chemother. 2015, 70, 1314–1321. [CrossRef]

8. CDC. Foodborne Diseases Active Surveillance Network. Available online: https://www.cdc.gov/foodnet/index.html (accessed on 21 October 2021).

9. EFSA-2021. Campylobacter and Salmonella Cases Stable in EU. Available online: https://www.efsa.europa.eu/en/news/ (accessed on 21 October 2021).

10. CDC. Foodborne Diseases Active Surveillance Network. Available online: https://www.cdc.gov/foodnet/index.html (accessed on 21 October 2021).

11. CDC. Foodborne Diseases Active Surveillance Network (FoodNet) Fast. Available online: https://wwwn.cdc.gov/foodnetfast/ (accessed on 21 October 2021).

12. Berden, J.H.; Muytjens, H.L.; van de Putte, L.B. Reactive arthritis associated with Campylobacter jejuni enteritis. Br. Med. J. 1979, 1, 380–381. [CrossRef] [PubMed]

13. Speed, B.; Kaldor, J.; Cavanaagh, P. Guillain-Barre syndrome associated with Campylobacter jejuni enteritis. J. Infect. 1984, 8, 85–86. [CrossRef]

14. Boyanova, L.; Gergova, G.; Spassova, Z.; Koumanova, R.; Yaneva, P.; Mitov, I; Derejian, S.; Krastev, Z. Campylobacter infection in 682 bulgarian patients with acute enterocolitis, inflammatory bowel disease, and other chronic intestinal diseases. Diagn. Microbiol. Infect. Dis. 2004, 49, 71–74. [CrossRef] [PubMed]

15. Newman, A.; Lambert, J.R. Campylobacteriosis. Available online: https://www.cdc.gov/foodnetfast/ (accessed on 21 October 2021).

16. Berden, J.H.; Muytjens, H.L.; van de Putte, L.B. Reactive arthritis associated with Campylobacter jejuni enteritis. Br. Med. J. 1979, 1, 380–381. [CrossRef] [PubMed]

17. Speed, B.; Kaldor, J.; Cavanaagh, P. Guillain-Barre syndrome associated with Campylobacter jejuni enteritis. J. Infect. 1984, 8, 85–86. [CrossRef]

18. Boyanova, L.; Gergova, G.; Spassova, Z.; Koumanova, R.; Yaneva, P.; Mitov, I; Derejian, S.; Krastev, Z. Campylobacter infection in 682 bulgarian patients with acute enterocolitis, inflammatory bowel disease, and other chronic intestinal diseases. Diagn. Microbiol. Infect. Dis. 2004, 49, 71–74. [CrossRef] [PubMed]

19. Newman, A.; Lambert, J.R. Campylobacteriosis. Available online: https://www.cdc.gov/foodnetfast/ (accessed on 21 October 2021).

20. Berden, J.H.; Muytjens, H.L.; van de Putte, L.B. Reactive arthritis associated with Campylobacter jejuni enteritis. Br. Med. J. 1979, 1, 380–381. [CrossRef] [PubMed]

21. Speed, B.; Kaldor, J.; Cavanaagh, P. Guillain-Barre syndrome associated with Campylobacter jejuni enteritis. J. Infect. 1984, 8, 85–86. [CrossRef]

22. Boyanova, L.; Gergova, G.; Spassova, Z.; Koumanova, R.; Yaneva, P.; Mitov, I; Derejian, S.; Krastev, Z. Campylobacter infection in 682 bulgarian patients with acute enterocolitis, inflammatory bowel disease, and other chronic intestinal diseases. Diagn. Microbiol. Infect. Dis. 2004, 49, 71–74. [CrossRef] [PubMed]

23. Newman, A.; Lambert, J.R. Campylobacteriosis. Available online: https://www.cdc.gov/foodnetfast/ (accessed on 21 October 2021).

24. Berden, J.H.; Muytjens, H.L.; van de Putte, L.B. Reactive arthritis associated with Campylobacter jejuni enteritis. Br. Med. J. 1979, 1, 380–381. [CrossRef] [PubMed]

25. Speed, B.; Kaldor, J.; Cavanaagh, P. Guillain-Barre syndrome associated with Campylobacter jejuni enteritis. J. Infect. 1984, 8, 85–86. [CrossRef]

26. Boyanova, L.; Gergova, G.; Spassova, Z.; Koumanova, R.; Yaneva, P.; Mitov, I; Derejian, S.; Krastev, Z. Campylobacter infection in 682 bulgarian patients with acute enterocolitis, inflammatory bowel disease, and other chronic intestinal diseases. Diagn. Microbiol. Infect. Dis. 2004, 49, 71–74. [CrossRef] [PubMed]
27. Subramanian, S.; Huq, S.; Yatsunenko, T.; Haque, R.; Mahfuz, M.; Alam, M.A.; Benezza, A.; DeStefano, J.; Meier, M.F.; Muegge, B.D.; et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 2014, 510, 417–421. [CrossRef]

28. Belkaid, Y.; Hand, T.W. Role of the microbiota in immunity and inflammation. *Cell* 2014, 157, 121–141. [CrossRef] [PubMed]

29. Caruso, R.; Lo, B.C.; Nunez, G. Host-microbiota interactions in inflammatory bowel disease. *Nat. Rev. Immunol.* 2020, 20, 411–426. [CrossRef] [PubMed]

30. Sun, X.; Winglee, K.; Gharaipeh, R.Z.; Gauthier, J.; He, Z.; Tripathi, P.; Avram, D.; Bruner, S.; Fodor, A.; Jobin, C. Microbiota-Derived Metabolic Factors Reduce Campylobacteriosis in Mice. *Gastroenterology* 2018, 154, 1751–1763.e1752. [CrossRef] [PubMed]

31. Han, Z.; Willer, T.; Li, L.; Pielsicker, C.; Rychlik, I.; Velge, P.; Kaspers, B.; Rautenschlein, S. Influence of the Gut Microbiota Composition on *Clostridium jejuni* Colonization in Chickens. *Infect. Immun.* 2017, 85, e00380–17. [CrossRef]

32. Deng, W.; Dittoe, D.K.; Pavilidis, H.O.; Chaney, W.E.; Yang, Y.; Ricke, S.C. Current Perspectives and Potential of Probiotics to Limit Foodborne *Campylobacter* in Poultry. *Front. Microbiol.* 2020, 11, 583429. [CrossRef] [PubMed]

33. Fu, Y.; Alenezi, T.; Almansour, A.; Wang, H.; Jia, Z.; Sun, X. The Role of Immune Response and Microbiota on Campylobacteriosis. In *Campylobacter*; IntechOpen: London, UK, 2021. [CrossRef]

34. Scupham, A.J.; Patton, T.G.; Bent, E.; Bayles, D.O. Comparison of the cecal microbiota of domestic and wild turkeys. *Microb. Ecol.* 2008, 56, 322–331. [CrossRef]

35. Kers, J.G.; Velkers, F.C.; Fischer, E.A.J.; Hermes, G.D.A.; Stegeman, J.A.; Smidt, H. Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. *Front. Microbiol.* 2018, 9, 235. [CrossRef] [PubMed]

36. Costa, M.C.; Bessegatto, J.A.; Alfieri, A.A.; Weese, J.S.; Filho, J.A.; Oba, A. Different antibiotic growth promoters induce specific changes in the cecal microbiota of broiler chickens. *PLoS ONE* 2017, 12, e0171642. [CrossRef]

37. Alrubaye, B.; Abraha, M.; Almansour, A.; Bansal, M.; Wang, H.; Kwon, J.M.; Huang, Y.; Hargis, B.; Sun, X. Microbial metabolite deoxycholic acid shapes microbiota against *Campylobacter jejuni* chicken colonization. *PLoS ONE* 2019, 14, e0214705. [CrossRef]

38. Shane, S.M. The significance of *Campylobacter jejuni* infection in poultry: A review. *Avian Pathol.* 1992, 21, 189–213. [CrossRef] [PubMed]

39. Chang, C.; Miller, J.F. *Campylobacter jejuni* colonization of mice with limited enteric flora. *Infect. Immun.* 2006, 74, 5261–5271. [CrossRef] [PubMed]

40. Cammarota, G.; Ianiro, G.; Gasbarrini, A. Fecal microbiota transplantation for the treatment of *Clostridium difficile* infection: A systematic review. *J. Clin. Gastroenterol.* 2014, 48, 693–702. [CrossRef] [PubMed]

41. Pang, X.; Hua, X.; Yang, Q.; Ding, D.; Che, C.; Cui, L.; Jia, W.; Bucheli, P.; Zhao, L. Inter-species transplantation of gut microbiota from human to pigs. *ISME J.* 2007, 1, 156–162. [CrossRef]

42. Wei, S.; Morrison, M.; Yu, Z. Bacterial census of poultry intestinal microbiome. *Poult. Sci.* 2013, 92, 671–683. [CrossRef]

43. Islam, K.B.; Fukiya, S.; Hagio, M.; Fujii, N.; Ishizuka, S.; Ooka, T.; Ogura, Y.; Hayashi, T.; Yokota, A. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 2011, 141, 1773–1781. [CrossRef] [PubMed]

44. Sakaridis, I.; Ellis, R.J.; Cawthraw, S.A.; van Vliet, A.H.M.; Stekel, D.J.; Penell, J.; Chambers, M.; La Ragione, R.M.; Cook, A.J. Investigating the Association Between the Caecal Microbiomes of Broilers and *Campylobacter* Composition on *Clostridium difficile* colonization of mice with limited enteric flora. *Infect. Immun.* 2020, 89, 927. [CrossRef] [PubMed]

45. Sato, Y.; Atarashi, K.; Plichta, D.R.; Arai, Y.; Sasajima, S.; Kearney, S.M.; Suda, W.; Takeshita, K.; Sasaki, T.; Okamoto, S.; et al. Novel bile acid biosynthetic pathways are enriched in the microbiome of centenarians. *Nature* 2021, 1–7. [CrossRef]

46. Baffoni, L.; Gaggia, F.; Garofolo, G.; Di Serafino, G.; Buglione, E.; Di Giannatale, E.; Di Gioia, D. Evidence of *Campylobacter jejuni* reduction in broilers with early synbiotic administration. *Int. J. Food Microbiol.* 2017, 251, 41–47. [CrossRef]

47. Augustinos, A.A.; Kyritsis, G.A.; Papadopoulos, N.T.; Abd-Alla, A.M.; Caceres, C.; Bourtzis, K. Exploitation of the Medfly Gut Microbiota for the Enhancement of Sterile Insect Technique: Use of *Enterobacter* sp. in Larval Diet-Based Probiotic Applications. *PLoS ONE* 2015, 10, e0136459. [CrossRef]

48. Lowenthal, A.; Livni, G.; Amir, J.; Samra, Z.; Ashkenazi, S. Secondary bacteremia after rotavirus gastroenteritis in infancy. *Pediatrics* 2006, 117, 224–226. [CrossRef] [PubMed]

49. Bar-Oz, B.; Preminger, A.; Peleg, O.; Block, C.; Arad, I. Enterobacter sakazakii infection in the newborn. *Acta Paediatr.* 2001, 90, 356–358. [CrossRef] [PubMed]

50. Mon, K.K.; Saelao, P.; Halstead, M.M.; Chanthavixay, G.; Chang, H.C.; Garas, L.; Suesu, M.A.; Zhou, H. Salmonella enterica Serovars Enteritidis Infection Alters the Indigenous Microbiota Diversity in Young Layer Chicks. *Front. Vet. Sci.* 2015, 2, 61. [CrossRef] [PubMed]

51. Wang, H.; Latorde, J.R.; Bansal, M.; Abraha, M.; Al-Rubaye, B.; Tellez-Issaia, G.; Hargis, B.; Sun, X. Microbial metabolite deoxycholic acid controls *Clostridium perfringens*-induced chicken necrotic enteritis through attenuating inflammatory cyclooxygenase signaling. *Sci. Rep.* 2019, 9, 14541. [CrossRef] [PubMed]

52. Fu, Y.; Almansour, A.; Bansal, M.; Alenezi, T.; Alrubaye, B.; Wang, H.; Sun, X. Microbiota attenuates chicken transmission-exacerbated campylobacteriosis in IL10(−/−) mice. *Sci. Rep.* 2020, 10, 20841. [CrossRef] [PubMed]

53. Bansal, M.; Fu, Y.; Alrubaye, B.; Abraha, M.; Almansour, A.; Gupta, A.; Liyanage, R.; Wang, H.; Hargis, B.; Sun, X. A secondary bile acid from microbiota metabolism attenuates ileitis and bile acid reduction in subclinical necrotic enteritis in chickens. *J. Anim. Sci. Biotechnol.* 2020, 11, 37. [CrossRef] [PubMed]
54. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]

55. Bacchetti De Gregoris, T.; Aldred, N.; Clare, A.S.; Burgess, J.G. Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J. Microbiol. Methods* **2011**, *86*, 351–356. [CrossRef]