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Age-based host response to turkey arthritis reovirus in commercial turkeys in the presence of maternally derived antibodies

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Abstract

Background Turkey arthritis reovirus (TARV) causes arthritic lameness in market-age turkeys. Since 2011, highly pathogenic TARV strains have caused significant economic losses in the turkey industry due to increased culling, reduced market weights, and decreased carcass quality, necessitating more effective control measures. Autogenous vaccine prevention strategies have been inefficacious partly due to a limited understanding of age-related susceptibility of turkeys to TARV. This study investigated age-related host and gut microbiota responses to TARV infection in commercial turkeys derived from vaccinated breeder hens. Poults with known maternally derived antibody titers were orally challenged with TARV O'Neil strain at 1-, 3-, and 7- weeks of age (WOA) and monitored for cloacal virus shedding, gastrocnemius tendon viral tropism, tendon inflammation, weight gain, and changes in gut microbiota.

Results A transient TARV-induced weight gain suppression was evident in poults infected at 1- and 3- WOA during the first 3 weeks post-infection. Age-dependent variations in cloacal viral shedding, virus isolation from tendons, and tendon inflammation severity were also observed. There was significant dissimilarity in ileal and cecal bacterial communities between mock and infected groups, but the effect of age of infection was unclear.

Conclusions Age dependent host response was observed to TARV based on cloacal virus shedding, weight gain suppression and viral tendon tropism. Our study also indicates that maternally derived antibodies appeared insufficient to prevent virus translocation to the tendons and subsequent pathological changes. This study lays the groundwork for future investigations of better vaccines/vaccination strategies and alternative preventive measures.

Importance Turkey arthritis reovirus (TARV) causes lameness due to arthritis and tenosynovitis, commonly in market-age turkeys, resulting in significant economic losses. As a control strategy, the turkey industry used autogenous vaccines, prepared from field TARV isolates in breeder hens, to protect the poults in the early stage of life through maternally derived antibodies (MDAs). This study establishes the level of protection provided by MDAs in young poults with age-based responses to TARV O'Neil reovirus strain. Additionally, this study reveals the dynamics of gut dysbiosis in infected poults at different timepoints, paving the way to ground-breaking investigations into gut

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microbiome modulation interventions that could potentially improve vaccine efficacy and reduce virus transmission and disease severity.

Background

In the United States, 218 million turkeys were raised in 2023 making the turkey industry a multi-billion-dollar industry [1]. The turkey industry has been facing several disease challenges, with reoviral arthritis/tenosynovitis being one of the most prevailing challenges in recent years [2, 3]. Turkey reoviral arthritis is caused by turkey arthritis reovirus (TARV) afflicting market aged turkeys resulting in lameness, hock joint swelling, valgus, and weight gain suppression [4–10]. TARV is causing significant economic losses to the turkey industry mainly due to culling and condemnation, reduced carcass quality, and reduced market weights. Based on report from National Turkey Federation, in 2019, TARV caused an estimated $33.7 million loss to industry [3, 11].

As a control strategy, breeder flocks are being immunized with autogenous vaccines, prepared from inactivated TARVs isolated from field cases [2, 12]. However, there has been an increase in the number of cases in recent years despite these vaccination efforts [12]. Vaccine failure is thought to be due to mutation of TARV strains circulating in the field [12–17]. Virus neutralization assay results strengthen this hypothesis as TARVs isolated from 2014 cases were found to be antigenically different from 2011 isolates and were unable to be completely cross neutralized [12]. Information about the extent of protection provided by maternally derived antibodies (MDAs) derived from breeder hens vaccinated with autogenous vaccines is lacking. Additionally, the frequent mutation of avian reoviruses and emergence of new strains will continue to hamper the efficacy of vaccination procedures and require constant vaccine modifications [18–23].

Moreover, while there is a clear link between age and susceptibility of chickens to virus infections, including reoviruses [24–29], age-related susceptibility to TARVs is not fully understood. Understanding of age-related susceptibility to TARVs is critical to developing efficacious control strategies against infection and design of post-infection intervention strategies to maintain optimal production performance and profitability. Using specific-pathogen-free (SPF) turkeys infected at 1 and 7 days of age, Ngunjiri and colleagues could not clearly demonstrate an age-dependent susceptibility to TARV infection or the development of reoviral arthritis feature such as hock joint swelling, tenosynovitis, distal tibiotarsal cartilage erosion, and lameness [10]. Kumar and colleagues later conducted a similar experiment by orally inoculating TARV into commercial turkeys at 2, 7, 14, 21 and 28 days of age [30]. The commercial turkeys across all age groups showed no significant variation in TARV replication and transmission to sentinels or histologic lesion scores in the gastrocnemius tendons, though birds inoculated at 28 days of age had slightly lower histologic scores [30]. The infection/transmission dynamics and subsequent development of arthritis have not been explored beyond 4 weeks of age.

Before translocating to the hock joint tendons, TARVs initially replicate in the epithelial cells of the gut mucosae that are colonized with commensal microbiota. Work by Ngunjiri and colleagues showed that influenza A virus infection in turkeys results in respiratory and enteric bacterial dysbiosis correlating with cytokine gene expression [31]. Such dysbiosis can enrich for coinfecting pathogens or negatively affect nutrient uptake and feed conversion ratios. While data from our initial study indicated that TARV infection is associated with dysbiosis of core gut microbiota in SPF turkeys [32], it remained unknown whether the severity of gut microbial dysbiosis is dependent on the age at virus exposure. Knowledge on age-dependent severity in dysbiosis is needed to inform the design probiotic candidates to modulate gut microbiota and reverse dysbiosis to possibly reduce disease severity and improve performance metrics post-infection.

It is critical to understand how transmission of TARV occurs in poults because TARV-induced clinical arthritis starts at around 8 weeks of age with peak symptoms around 12–17 weeks of age. Also, the extent of protection of young poults against novel highly pathogenic TARV strains by MDAs needs to be understood to help design or optimize current vaccination strategies.

Results

Maternally derived antibodies (MDAs) waned by 3 weeks of age

As the breeder hens were vaccinated with autogenous vaccines prepared from field isolates of TARVs, the poults possessed high MDA titers of at 1 day of age (mean = 1972) (Fig. 1). The average MDA titers subsequently decreased to 166.9 at 1 WOA, 17.02 at 3 WOA, and 2.12 at 7 WOA.

TARV induced weight gain suppression was pronounced from 0 to 1 week post infection

Turkey reoviral arthritis induces reduced weight gain mainly due to enteritis during early phase of infection and arthritic symptoms during late phase of infection [7, 10, 30]. Figure 2A shows percentage weight gain from 0 to 1 WPI and 2 to 3 WPI. Each inoculated group was compared with the mock control group of the same age.

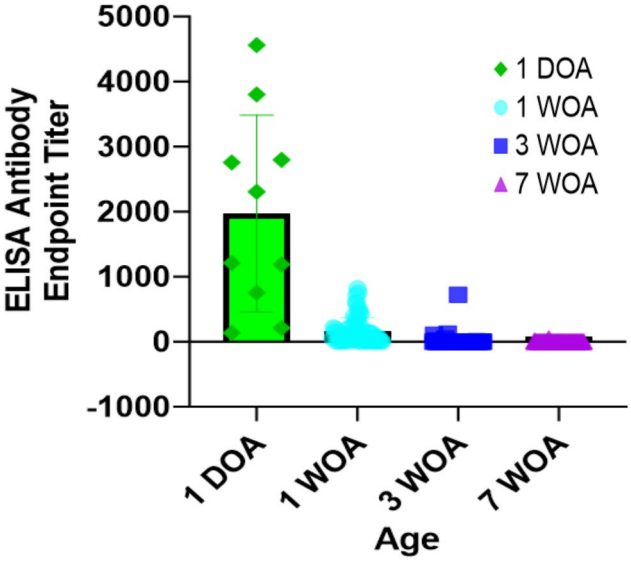

Fig. 1 Maternal derived antibodies on 1 DOA, 1, 3 and 7 WOA before inoculation with virus. ELISA endpoint titers. Birds were inoculated with virus-free growth medium (Mock) or TARV O'Neil at 1, 3 and 7 weeks of age. DOA = days of age. WOA = weeks of age

Significant weight gain suppression was observed from 0 to 1 WPI in poults inoculated at 1 WOA ($p < 0.0001$) and 3 WOA ($p = 0.0305$), but not those inoculated at 7 WOA ($p = 0.0694$). From 2 to 3 WPI, gain suppression was reversed: all inoculated groups gained weight more rapidly compared to age-matched Mock controls ($p = 0.0108$, $p < 0.0001$, $p < 0.0001$ for 1-, 3-, and 7- WOA inoculates, respectively). It should be noted that that despite the reversal of the weight gain suppression from 2 to 3 WPI, the absolute weights of inoculated poults were significantly lower compared to the age matched controls (Table 1). Overall, the effect of weight gain suppression was higher in poults for 1 WOA inoculates in comparison to 3 and 7 WOA inoculates from 0 to 1 WPI.

Cloacal viral shedding was significantly higher in poults infected at 1- and 3- weeks of age

Previous studies have shown that the cloacal shedding for TARV appeared to decline significantly over time with no shedding by most poults at 2 WPI [6, 7, 10]. Therefore, we analyzed virus shedding at 3 and 7 DPI for all groups. Cloacal viral shedding was significantly higher at 3- and 7- DPI in poults infected at 1 week of age compared to poults infected at 7 weeks of age (Fig. 2B). The $\log_{10}$ viral titers were highest in 1 WOA inoculates at 3 DPI (mean = 6.329 $\log_{10}$ GE/ml) and lowest in 7 WOA inoculates at 7 DPI (mean = 0.262 $\log_{10}$ GE/ml).

The tendon tropism of virus was higher in poults infected at 1 week of age

After oral inoculation, the virus replicates in the intestines, then migrates to tendons and other organs including liver and spleen [10, 33]. Our previous study with SPF turkeys inoculated at 1 WOA demonstrated the tendon tropism to be maximum at 4 WPI [10]. Therefore, we focused on this time point for virus detection via quantitative RT-PCR (RT-qPCR) and isolation. The average viral titer was numerically higher in poults infected at 1 WOA (mean = 4.53 $\log_{10}$ GE/g) compared to other groups (3 WOA = 2.18 $\log_{10}$ GE/g; 7 WOA = 1.95 $\log_{10}$ GE/g), however, there was no significant differences (Fig. 3A).

Although our RT-qPCR assay is significantly more sensitive than previously published assays and can detect a few TARV gene copies [10, 34], its accuracy is subject to the quality of the RNA being analyzed and the practicality of obtaining a tendon sample that truly represents the overall virus distribution in the hock joint tendons. Therefore, all tendon samples from the infected groups were subjected to three blind passages in QT-35 cells to isolate the virus. TARV was isolated from gastrocnemius and digital flexor tendon from all 6 poults infected at 1 WOA (Fig. 3B). However, live virus was isolated from only one out of six poults infected at 3 WOA and none of the poults infected at 7 WOA.

Histologic inflammation scores of gastrocnemius tendon sheaths were numerically higher in poults infected at 1 week of age

The severity of inflammation of gastrocnemius tendon in hock joints was scored by a pathologist who was blinded to treatment using a previously published method [7]. It has been observed that histologic demonstration of lymphocytic tenosynovitis tends to increase from 7 DPI, peak at 3–4 WPI and decrease by 15 WPI [7, 10]. Additionally, the tenosynovitis score at 4 WPI can be correlated with occurrence of lameness in later stage of life generally appearing at 8 WOA [7]. At 4 WPI, the inflammation score was numerically high in poults infected at 1 week of age (mean = 4.2) compared to poults infected at 3 (mean = 3.5) and 7 weeks of age (mean = 3.5) (Fig. 3C). When compared to mock, the scores were significantly higher ($p < 0.0001$) in poults infected at 1 and 3 WOA; while there was no significant difference ($p = 0.29$) in scores in poults infected at 7 WOA, when compared to age-matched mock groups.

Gut microbiota changes were observed between mock and TARV inoculated groups

Principal Coordinate Analysis (PCoA) plots showing Beta diversity of the cecum and ileum microbial community are shown in Fig. 4A and B, respectively. The significance of differences between mock and infected groups were computed by PERmutational Multivariate ANalysis of VAriance (PERMANOVA) test in R using the vegan package (Table 2). For cecum, significant differences

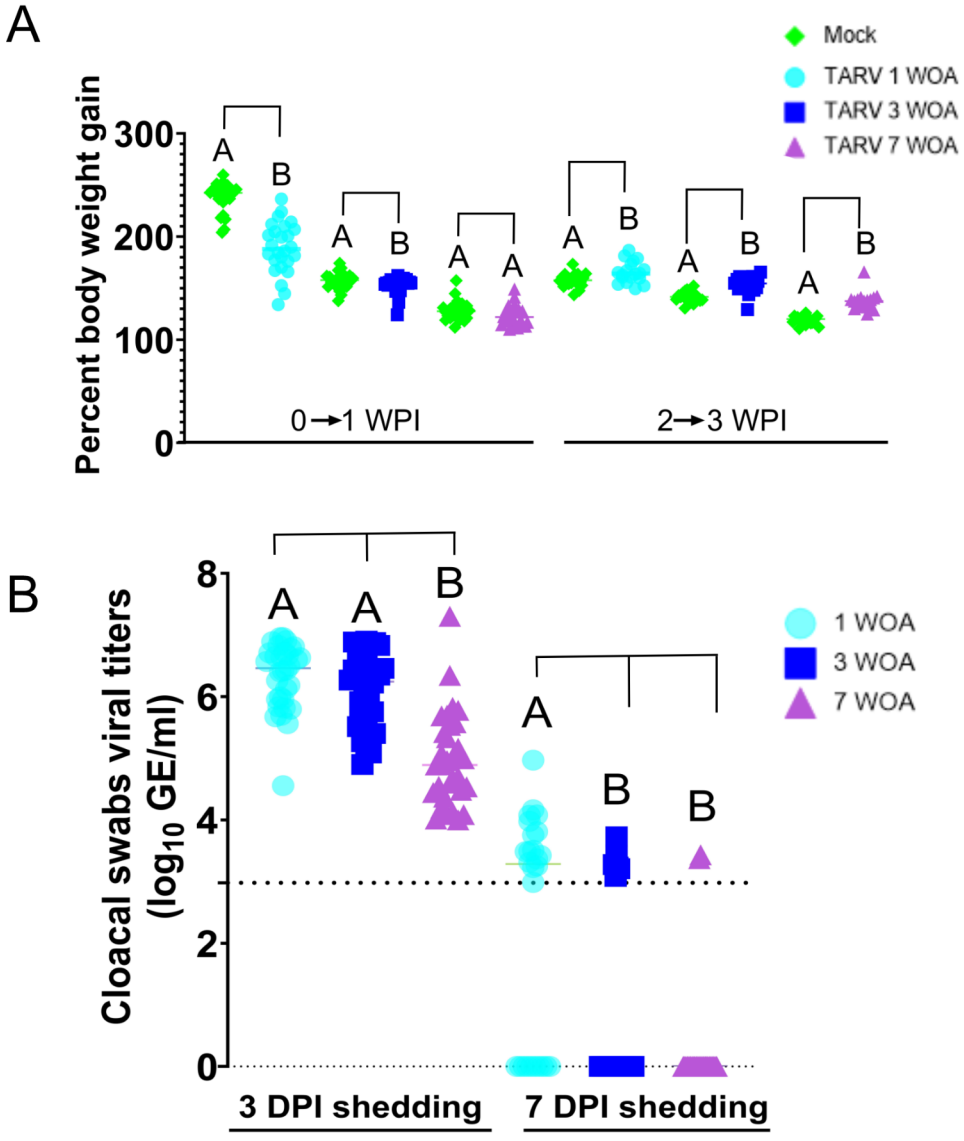

Fig. 2 Weight gain suppression and cloacal viral shedding in inoculated groups. (**A**) Percent weight gain observed among mock and inoculated groups from 0 to 1 WPI and 2 to 3 WPI. (**B**) Cloacal viral shedding at 3 DPI and 7 DPI. Viral titers were extrapolated from cycle threshold (Ct) values obtained through qRT-PCR of the S4 gene segment. Statistical comparisons were performed between mock and inoculated groups within age groups using Student's t-test with Welch's correction ($p < 0.05$, different letters in each group indicate significant differences). DPI = days post infection. WPI= weeks post infection. WOA = weeks of age

($p < 0.008$) were observed between mock and infected groups at all times post infection for all inoculates (1, 3, and 7 WOA). For ileum, significant differences were observed between mock and infected groups at all times post infection for 1 WOA ($p < 0.004$) inoculates, at 3 DPI ($p = 0.013$) and 7 DPI ($p = 0.003$) for 3 WOA inoculates, and at 4 WPI ($p = 0.016$) for 7 WOA inoculates.

Alpha diversity (using the Shannon index) was computed using the R/Bioconductor package phyloseq and the significance of differences between mock and inoculated groups (Fig. 5A and B) was determined using Wilcoxon rank sum tests with adjusted p-value (Table 3). No significant differences were observed for bacterial richness between mock and infected groups for all groups except at 4 WPI ($p = 0.041$) in ileum for 7 WOA inoculates.

The abundance plot depicts the difference in total abundance of cecal and ileal bacterial families and genera. Figures 6 and 7 show the pooled abundance of samples from each group for bacterial families and genera and supplementary figures S1 and S2 depict the abundance of individual samples for different groups for bacterial families and genera.

At the family level, the most abundant family in cecum was Lachnospiraceae in mock groups, while we observed a transition to Enterobacteriaceae and Ruminococcaceae

Table 1 Absolute weights at different time post inoculation. (p value, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, NS = non-significant)

		Mock			Infected				
Group	**Days post infection**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**P value**	**Significance level**
1WOA	0 DPI	175.9	13.17	32	164	19	32	0.0059	**
1WOA	1 WPI	418.7	41.16	26	311.2	40.18	26	< 0.0001	****
1WOA	2 WPI	764	93	20	579.4	66.13	18	< 0.0001	****
1WOA	3 WPI	1205	152.8	20	959	126	18	< 0.0001	****
3 WOA	0 DPI	580.5	9.381	32	690.2	85.57	32	0.0686	NS
3 WOA	1 WPI	1214	157	25	925.1	123.6	26	< 0.0001	****
3 WOA	2 WPI	1668	203.4	20	1309	169	20	< 0.0001	****
3 WOA	3 WPI	2357	257.7	20	2005	224.1	20	< 0.0001	****
7 WOA	0 DPI	3202	508	32	2659	336.6	32	< 0.0001	****
7 WOA	1 WPI	4165	422.3	26	3223	443	26	< 0.0001	****
7 WOA	2 WPI	5570	469.2	20	3840	513.4	20	< 0.0001	****
7 WOA	3 WPI	6630	504	20	5195	582.6	19	< 0.0001	****

in infected groups for 1 WOA inoculates (Fig. 6A). However, the most abundant family in cecum was Lachnospiraceae for both mock and infected groups for 3 WOA inoculates at all time post infection (Fig. 6B). The most abundant genera in cecum were *Lachnoclostridium* (3 DPI), Lachnospiraceae NK4A136 group (7 DPI) and *Ruminococcus* (4 WPI) in mock groups, while we observed a transition to Escherichia-Shigella (3 DPI), and *Streptococcus* (7 DPI) in infected groups for 1 WOA inoculates (Fig. 6C). However, the most abundant genera in cecum were *Ligilactobacillus* (3DPI), *Anaerostipes* (7 DPI) and *Ruminococcus* (4 WPI) for mock groups for 3 WOA (Fig. 6D). A transition to *Ruminococcus* (3 DPI and 7 DPI) and *Faecalibacterium* (4 WPI) was observed in infected 3 WOA inoculates.

For ileum, the most abundant families were Peptostreptococcoceae (3 DPI and 7 DPI) and Erysipelotrichaceae (4 WPI) in mock groups, while they were Clostridiaceae (3 DPI and 7 DPI) and Peptostreptococcoceae (4 WPI) in infected groups for 1 WOA inoculates (Fig. 7A). We observed Lactobacillaceae (3 DPI and 4 WPI) and Peptostreptococcaceae (7 DPI) as the most abundant family in mock groups while Lactobacillaceae (3 DPI, 7 DPI, and 4 WPI) in infected groups for 3 WOA inoculates (Fig. 7B). For ileum, the most abundant genera were *Rombutsia* (3DPI and 7 DPI) and *Turicibacter* (4WPI) in mock and *Clostridium* (3DPI and 7 DPI) and *Rombutsia* (4WPI) in infected groups for 1 WOA inoculates (Fig. 7C). Similarly, the most abundant genera in ileum were *Ligilactobacillus* (3DPI), *Rombutsia* (7DPI) and *Labctobacillus* (4WPI) for mock groups while the transition to *Ligilactobacillus* (3DPI and 7 DPI) and *Turicibacter* (4WPI) in infected groups for 3 WOA inoculates (Fig. 7D).

Discussion

Outbreaks of turkey reoviral arthritis are causing significant losses to the turkey industry and the virulence and tissues tropisms appear to be changing [3]. As a prevention strategy, the turkey industry has been vaccinating breeder hens with autogenous vaccines, which are prepared from field isolates of TARVs [2]. The autogenous vaccine used in breeder hens, from where the poults used in this study were derived, was prepared from 4 TARVs isolated in Indiana, Ohio, and Pennsylvania. Even though ~ 10- to 80-fold higher MDA titers were detected in poults at 1 WOA (mean = 166.9) compared to 3 WOA (mean = 17.02) and 7 WOA (mean = 2.12), 1 WOA inoculates showed higher cloacal viral shedding at 3 DPI and 7 DPI compared 3 and 7 WOA inoculates. The non-protective nature of MDAs in this study might be due to differences in antigenicity between inoculated virus (TARV O'Neil) and the viruses used in autogenous vaccines. For example, TARV O'Neil was first isolated in 2010 while the recent outbreak viruses have been observed to have undergone genetic reassortment and mutation [5, 12–15, 35].

The protection window of reovirus MDAs in turkeys may be very short, likely less than one week after hatching. In our previous surveillance study of two turkey flocks, we observed high MDA titers at 1 WOA, which declined to low levels by 3 WOA [36]. The subsequent rise in reovirus-specific antibodies from the third WOA onward, observed in a previous surveillance study, indicated breakthrough infections with reovirus of unknown pathogenicity [36]. In contrast, MDAs have been effective in the control of chicken reovirus (CARV) [27, 37, 38]. Further research with various TARV strains is necessary to determine the effectiveness of MDAs and autogenous vaccines in controlling turkey reoviral arthritis.

As discussed above, MDA levels in turkey poults at time infection did not seem to impact virus replication.

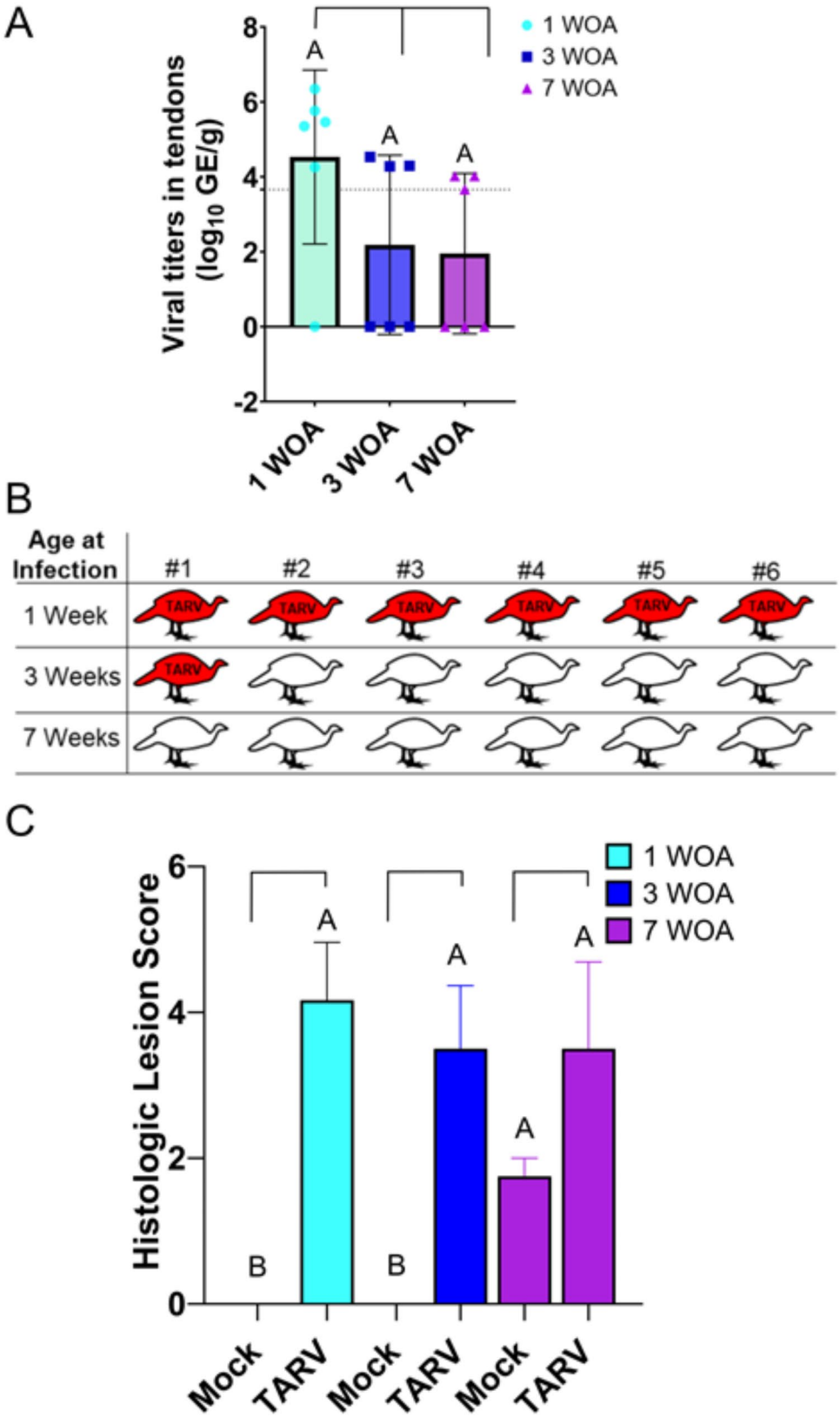

Fig. 3 (See legend on next page.)

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Fig. 3 Tendon viral titers in inoculated groups, virus isolation from tendons and histologic inflammation of tendon sheaths. (**A**) Tendon viral titers at 4 WPI. Viral titers were extrapolated from cycle threshold (Ct) values obtained through qRT-PCR of the S4 gene segment. Statistical comparisons were performed using one-way ANOVA followed by Tukey's test for pair-wise comparisons. (**B**) Virus isolation from gastrocnemius and digital flexor tendons at 4 WPI. (**C**) Histologic inflammation of gastrocnemius tendon sheaths. Statistical comparisons were performed between mock and inoculated groups within age groups using Student's t-test with Welch's correction ($p < 0.05$, different letters indicate significant differences). WPI= weeks post infection. WOA = weeks of age

Still, the viral titers in cloacal swabs decreased by about 100- to 1000- folds from 3DPI to 7 DPI consistently in all groups. This decrease is likely due to the action of innate immune responses such as antiviral cytokines in intestines [6, 7, 9, 10, 30, 33]. Furthermore, the consistently lower cloacal shedding in 7-week-old inoculates at both time points compared to 1- and 3-week-old inoculates is consistent with the age-dependent maturation of innate immune responses in avian species [39].

The tendon RT-qPCR viral titers and histologic lesion scores at 4 WPI were numerically higher but not statistically significant in 1 WOA inoculates compared to other groups and correlated with the rate of virus isolation from gastrocnemius and digital flexor tendons at 4 WPI. The high variability of RT-qPCR viral titers in tendon samples is likely due to practical limitations related to the quality of extracted RNA and uneven virus distribution in different sites of the hock joint tendon sheaths. Nonetheless, these results suggest that the tendons of 1 week-old or younger turkeys are more susceptible to TARV infection and subsequent development of tenosynovitis compared to older age groups. Kumar and colleagues described a similar pattern where poults 1 WOA or younger showed numerically higher tendon log viral gene copy numbers and tendon histologic lesion scores compared to those inoculated at older ages [30].

An age-based response of turkey poults to TARV infection may vary depending upon the pathogenicity of the infecting strain as observed with chicken reovirus. In chickens, age-based resistance was observed with highly pathogenic strains compared to low pathogenicity strains [24–26, 28, 40]. Age based resistance may be attributed to increasing ability of the immune response to prevent viral dissemination except at immune sequestered sites such as joints and tendons. Rosenberger et al. (1988) also showed age-based resistance in chicken based on body weight suppression, cloacal shedding, and antibody response with one day inoculates being higher susceptible compared to two week inoculates [25, 40].

The microbiome analysis showed ileal and cecal dysbiosis when compared between mock and inoculated groups as demonstrated by alpha and beta diversity. A similar trend of disruption in normal microbiota was found in influenza and hemorrhagic enteritis virus-infected turkey poults [31, 41]. Viral diseases have also been observed to cause intestinal bacterial dysbiosis in chickens, and ducks [42–46]. There is a change in bacterial composition in ileum and cecum due to age and other confounding factors in both turkeys and layer chickens [47–50].

The decrease in abundance of genus *Lactobacillus* in TARV infected groups suggests use of a host tailored probiotic such as *Lactobacillus* species might be beneficial in restoring the microbiome. However, the inconsistency in the abundance of genus *Lactobacillus* and Family Lactobacillaceae among mock and inoculated groups might be due to a smaller number of samples in each timepoint and group [51, 52].

Conclusions

In conclusion, we observed age-based susceptibility to turkey arthritis reoviral infection based on cloacal virus shedding, weight gain suppression, and viral tendon tropism. Maternally derived antibodies, derived from breeder hens vaccinated with autogenous vaccines, are likely insufficient to prevent TARV infection and associated pathology. This study demands further research on protection provided by maternally derived antibodies in poults when challenged with the same virus strain used for preparation of autogenous vaccines for breeder hens. Furthermore, the vaccination strategies for turkey reovirus could be optimized using virus vectored or nanoparticle subunit vaccines, which are successful in controlling various avian diseases in recent years [20, 21, 53–58].

Future studies should focus on studying the role of passive immunity in protecting TARV induced arthritis. Additionally, the contribution of intrinsic microbial factors towards onset, development, and severity of reovirus associated arthritis needs to be addressed. Furthermore, there is opportunity to investigate the utility of probiotics, specifically those belonging to genus *Lactobacillus*, in reversing gut dysbiosis, reducing severity of turkey reoviral arthritis [50], and enhancing vaccine efficacy.

Methods

Ethics statement

All husbandry and experimental procedures involving turkeys in this study were approved by The Ohio State University Institutional Animal Care and Use Committee under protocol #2011A000000109-R4. This protocol complies with the U.S. Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Ohio State University holds accreditation from the Association for the Assessment and

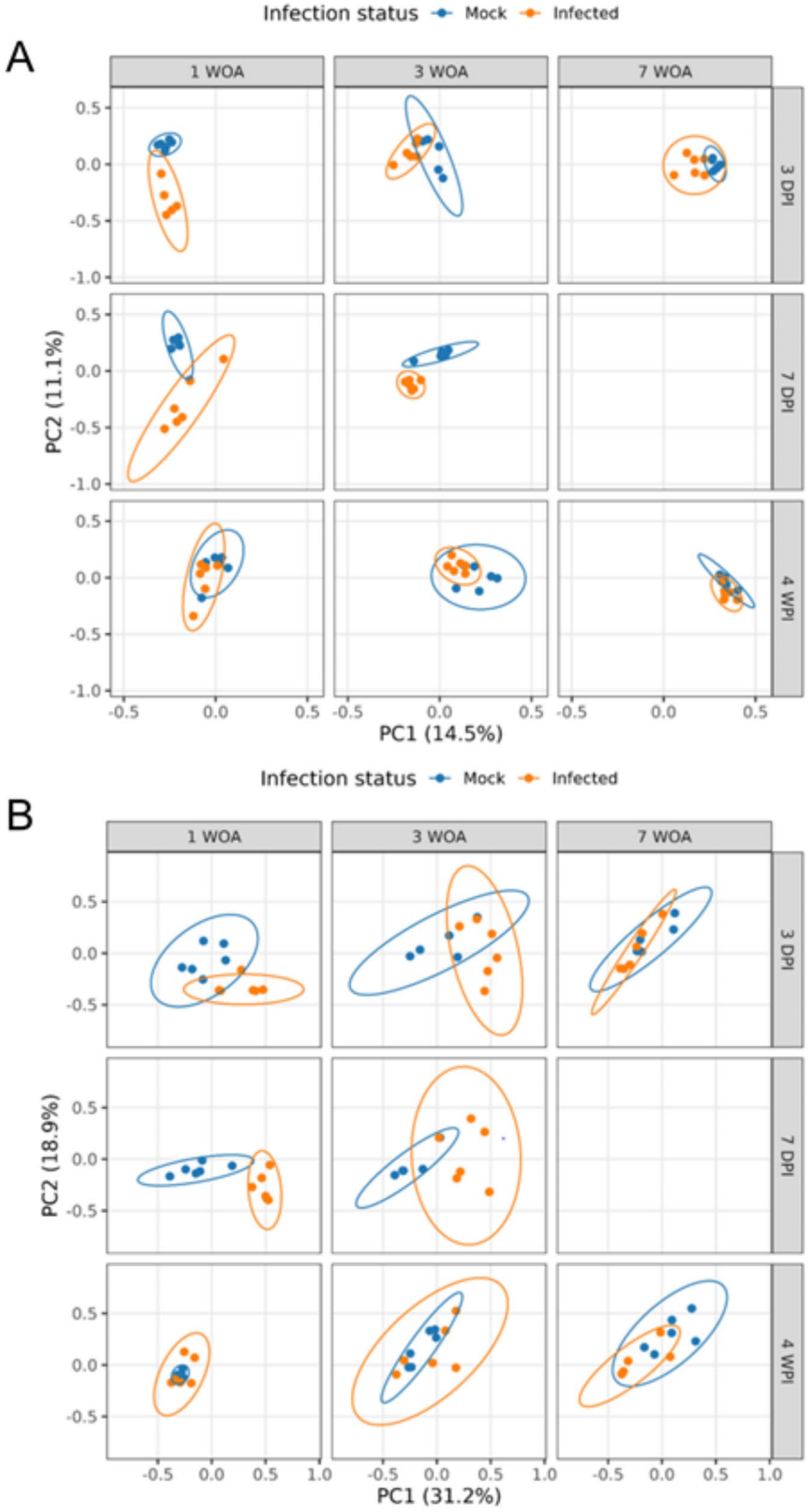

Fig. 4 Overall microbial community differences between mock and inoculated groups. (**A**) Principal Coordinate Analysis (PCoA) plot of cecal microbial community and and (**B**) ileal microbial community at 3 DPI, 7 DPI, and 4WPI of 1 WOA, 3 WOA, and 7 WOA inoculates. DPI = days post infection. WPI= weeks post infection. WOA = weeks of age

Table 2 Beta diversity analysis (PCA plot) of cecal and ileal bacterial communities between mock and infected groups with level of significance (PERMANOVA test). (p value, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, NS = non-significant)

S.No.	Group	Section	Time post inoculation	PERMANOVA test (*p*-value)	Significance level (*p* value)
1.	1 WOA	Cecum	3 DPI	0.003	**
2.	1 WOA	Cecum	7 DPI	0.002	**
3.	1 WOA	Cecum	4 WPI	0.003	**
4.	3 WOA	Cecum	3 DPI	0.003	**
5.	3 WOA	Cecum	7 DPI	0.006	**
6.	3 WOA	Cecum	4 WPI	0.001	***
7.	7 WOA	Cecum	3 DPI	0.008	**
8.	7 WOA	Cecum	4 WPI	0.003	**
9.	1 WOA	Ileum	3 DPI	0.004	**
10.	1 WOA	Ileum	7 DPI	0.003	**
11.	1 WOA	Ileum	4 WPI	0.001	***
12.	3 WOA	Ileum	3 DPI	0.013	*
13.	3 WOA	Ileum	7 DPI	0.003	**
14.	3 WOA	Ileum	4 WPI	0.082	NS
15.	7 WOA	Ileum	3 DPI	0.077	NS
16.	7 WOA	Ileum	4 WPI	0.016	*

Accreditation of Laboratory Animal Care International (AAALAC).

One-day-old turkey poults were obtained from a commercial hatchery in Ohio. These poults were derived from breeder hens vaccinated with an autogenous vaccine containing antigens from four turkey arthritis reovirus (TARV) isolates collected from Indiana, Ohio, and Pennsylvania. The hatchery owner fully consented to the use of these animals for research purposes and agreed to the publication of the resulting data but requested anonymity in the published work.

The turkeys were housed in a biosafety level 2 (BSL-2) animal research facility equipped with forced air ventilation and HEPA filters on all incoming and outgoing air to prevent ammonia buildup. The birds were raised on the floor with wood shavings litter, and room temperatures were maintained at 25 ± 3 °C. They were provided with ad libitum access to water and crumbled feed prepared at The Ohio State University's feed milling center at the Wooster campus. The same feed was used for all groups throughout the study and no antibiotics or bacterial growth-inhibiting substances were added to either feed or water during the study. Animal health and well-being were monitored twice daily throughout the study. The turkeys were observed for reovirus-associated clinical signs such as decreased activity, reluctance to moved when prodded, incoordination, tremors, twisted necks, twitches, dehydration, sunken eyes, ruffled feathers, diarrhea, respiratory distress, swollen hock joints, as well as injuries that were not related to experimental treatment.

Turkeys exhibiting signs of distress were humanely euthanized as soon as possible using carbon dioxide (CO_2) exposure. Depending on age and size, 1–10 animals were placed in a euthanasia chamber connected to a CO_2 source, with CO_2 flow set at 10–30% displacement of chamber volume per minute. Birds were monitored for respiratory arrest, and CO_2 flow continued for at least one minute after arrest was confirmed. Absence of breathing and heartbeat was verified; if any vital signs were detected, additional CO_2 exposure was administered. Following confirmed death, secondary physical euthanasia (cervical dislocation or removal of a vital organ) was performed before tissue collection and carcass disposal.

Viruses

TARV O'Neil (ON) was kindly provided by Dr. Jack Rosenberger. It was propagated in Japanese quail fibrosarcoma cell (QT-35). The virus was titrated for median tissue culture infectious dose ($TCID_{50}$) in quail fibrosarcoma cell (QT-35) on 96 well plates using the Reed and Muench method [59].

Experimental design

Six groups were divided randomly from a total of 191 one-day commercial turkey poults, with 32 poults per group (except 31 poults in one group) (Table 4). Higher number of poults were initially recruited in each group to cover the statistical power for body weight comparison at 2 and 3 WPI. The poults were swabbed and bled before inoculation. The swabs were processed and RTqPCR was performed indicating the absence of infection of TARV. Three groups of poults were orally inoculated with 4×10^6 median tissue culture infectious dose ($TCID_{50}$) of TARV O'Neil at 1, 3, and 7 weeks of age (WOA) and the other three groups were inoculated virus-free medium. The virus stocks were grown in antibiotic free medium, and the mock groups were also inoculated with antibiotic free

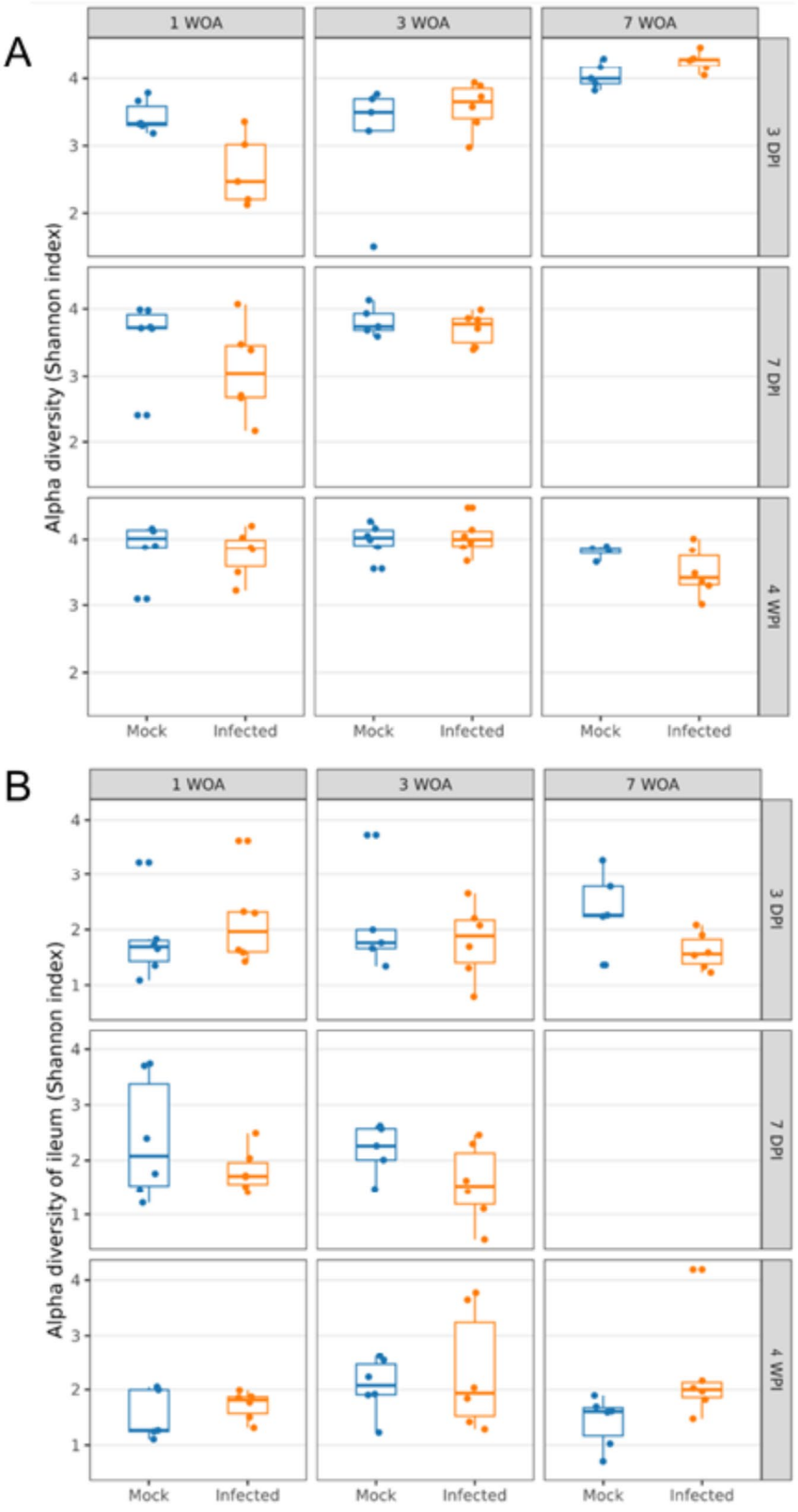

Fig. 5 Alpha diversity of ileum and cecum microbiota among mock and inoculated groups. (**A**) Alpha diversity (Shannon index) of cecal bacterial community (**B**) Alpha diversity (Shannon index) of ileal bacterial community at 3 DPI, 7 DPI and 4WPI of 1 WOA, 3 WOA and 7 WOA inoculates. DPI = days post infection. WPI= weeks post infection. WOA = weeks of age

Table 3 Alpha diversity (Shannon measures) of richness of bacterial communities between mock and infected groups with significance level (Wilcoxon rank sum test). (p value, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, NS = non-significant)

S.No.	Group	Section	Time post inoculation	Wilcoxon test (adjusted *p*-value)	Significance level
1.	1 WOA	Cecum	3 DPI	0.052	NS
2.	1 WOA	Cecum	7 DPI	0.24	NS
3.	1 WOA	Cecum	4 WPI	0.48	NS
4.	3 WOA	Cecum	3 DPI	0.43	NS
5.	3 WOA	Cecum	7 DPI	0.66	NS
6.	3 WOA	Cecum	4 WPI	1	NS
7.	7 WOA	Cecum	3 DPI	0.13	NS
8.	7 WOA	Cecum	4 WPI	0.26	NS
9.	1 WOA	Ileum	3 DPI	0.59	NS
10.	1 WOA	Ileum	7 DPI	0.18	NS
11.	1 WOA	Ileum	4 WPI	0.66	NS
12.	3 WOA	Ileum	3 DPI	0.93	NS
13.	3 WOA	Ileum	7 DPI	0.17	NS
14.	3 WOA	Ileum	4 WPI	1	NS
15.	7 WOA	Ileum	3 DPI	0.052	NS
16.	7 WOA	Ileum	4 WPI	0.041	*

medium. Six poults from each group were euthanized at 3-, 7- and 28- days post inoculation (DPI) respectively and cloacal swabs, hock joint, ileal, and cecal contents were collected. The birds were euthanized using gaseous CO_2. No cecal or ileal contents were collected for microbiome analysis at 7 DPI for 7 WOA inoculates.

Antibody detection

The antibody concentration was measured via ELISA using a commercial IDEXX Avian Reovirus kit (Maine, USA) according to manufacturer's instructions. The sample to positive ratio (S/P) ratio was converted into antibody endpoint titers using the formula provided in the protocol. Even though the assay was developed to detect chicken IgG, it has been sensitive in detecting serum antibodies induced by reovirus strains used in the previous and current studies [10].

Virus detection

RNA was extracted from cloacal swabs, tendon homogenates using RNeasy Mini Kit (QIAGEN). Quantitative RT-PCR (qRT-PCR) was performed via the amplification of a 145 bp fragment of S4 segment with following primers: Forward 5'-CATGATGGCGGCTCAACT-3' and 5'-CATCAGCTCACGATCAATAGG-3' [34, 60, 61]. The reactions were performed using a QuantiTect SYBR Green RT-PCR Kit (Qiagen Sciences Inc., MD, USA) using the following parameters: 50˚C for 30 min; 95˚C for 15 min; 45 cycles of 94˚C for 15 s, 61˚C for 60 s and 72˚C for 45 s; 95˚C for 15 sand 60˚C for 60s. Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, MA) and Roche LightCycler (Roche Diagnostics, Boston, MA) were used to perform reactions. PCR generated Ct values were extrapolated into genomic equivalent titers using a standard curve generated with viral RNA from the same stock used for infection [10].

Virus isolation

The hock joint was collected with a bone cutter. Using sterile forceps and scissors, both gastrocnemius and digital flexor tendons were secured. Around 350 mg of tendons were initially collected from each individual bird representing each group for virus isolation. The tendons were collected in 2 ml screw cap tubes containing phosphate buffered saline. They were chopped then homogenized using a Tissuelyzer II using parameters: 30 hz for 30 s, 30 hz for 1 min. Next the homogenized sample was centrifuged at 12,000 rpm for 1 min and supernatant was subjected to 6 well plates with confluent QT-35 cells. The reovirus specific cytopathic effect (CPE) was confirmed when there was presence of clumping, swelling and detachment of cells.

Staining

The hock joints were collected in 10% neutral buffered formalin, followed by decalcification in a 10% EDTA solution [62]. Next, they were fixed in paraffin and sectioned into 5 µm sections and then stained with Hematoxylin and Eosin (H & E) staining. Histologic inflammation was scored using a previously developed scoring system [7] by poultry pathologist who was blinded to the infection status of poults.

Microbiome samples

Ileum and cecum contents were taken aseptically during necropsy of euthanized poults and stored at -80˚C. Next, 0.2 gm of ileal and cecal content was used for DNA extraction using PureLink Microbiome DNA Purification Kit (ThermoFisher Scientific, USA). DNA quality and quantity was determined using NanoDrop Spectrophotometer (ThermoFisher Scientific). Samples were subjected to 16 S rRNA V4-V5 sequencing at Ohio State University's Molecular and Cellular Imaging Center (MCIC) in Wooster. A portion of this hypervariable region of the 16 S rRNA gene was PCR-amplified using primers GAGTGCCAGCMGCCGCGGTAA and ACGGACTACHVGGGTWTCTAAT. Next, Illumina libraries were generated using Nextera XT DNA Library Preparation Kit (Illumina, USA) followed by paired-end 300 bp sequencing on an Illumina (San Diego, CA, USA) MiSeq.

Raw sequences in demultiplexed FASTQ files were first subjected to quality control with FastQC (version 0.12.1) [63] and MultiQC (version 1.21) [64]. Next, PCR primers (and any adapters) were removed using Cutadapt (version

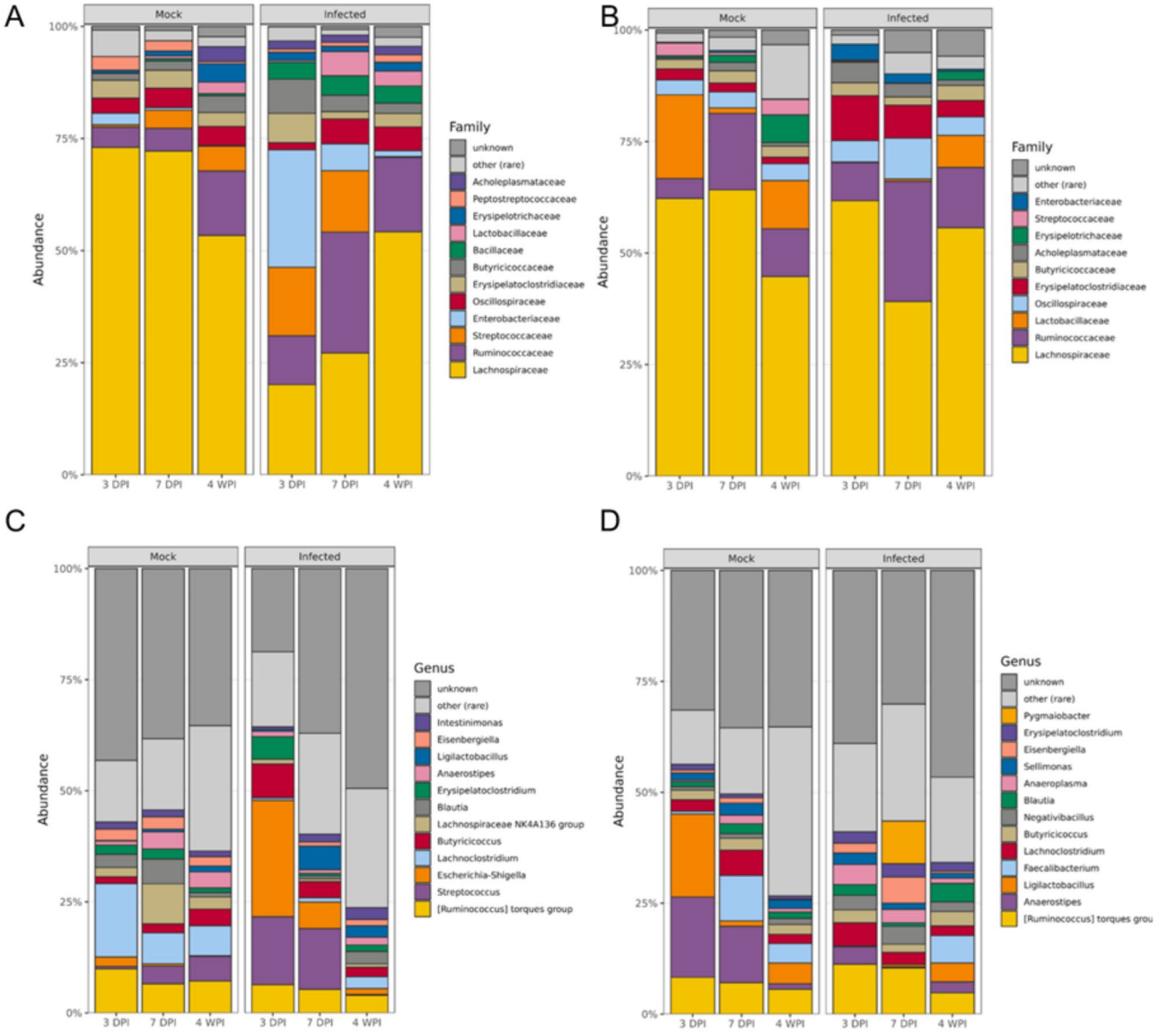

Fig. 6 Abundance of bacterial community members in cecum among mock and inoculated groups. Abundance of cecal bacterial composition at the family level for 1WOA (**A**) and 3 WOA (**B**) inoculates among mock and infected groups. Abundance of cecal bacterial composition at the genus level for 1WOA (C) and 3 WOA (D) inoculates among mock and infected groups. DPI = days post infection. WPI= weeks post infection. WOA = weeks of age

4.6) [65]. We then used a standardized series of steps implemented in the R/Bioconductor package DADA2 (version 1.30.0) [66] to filter and trim sequences, infer Amplicon Sequence Variants (ASVs), generate an ASV count table, and assign taxonomy to ASVs using the Silva database (version 138.1, available at https://zenodo.org/record/4587955) [67] as a reference. The ASV count table was filtered by [1] ASV length, retaining only ASVs with lengths between 250 and 190 bp; [2] taxonomy, removing ASVs that had been assigned to the order Chloroplast, the family Mitochondria, or the domain Eukaryota were removed; [3] sample ASV count, removing samples with a total ASV count below a 1,000. All bioinformatics analyses were performed at the Ohio Supercomputer Center (1987).

We inferred a phylogenetic tree for all ASVs in the final count table using the R package phangorn (version 2.11.1) [68]. The R/Bioconductor package phyloseq (version 1.41.1) [69] was used for most downstream analyses, such as computing beta (using Principal Coordinate Analysis) and alpha (using the Shannon diversity index) diversity. Differences in diversity were assessed using the Wilcoxon rank sum text for alpha diversity and the PERMANOVA as implemented in the R package vegan (version 2.6-4) [70] for beta diversity.

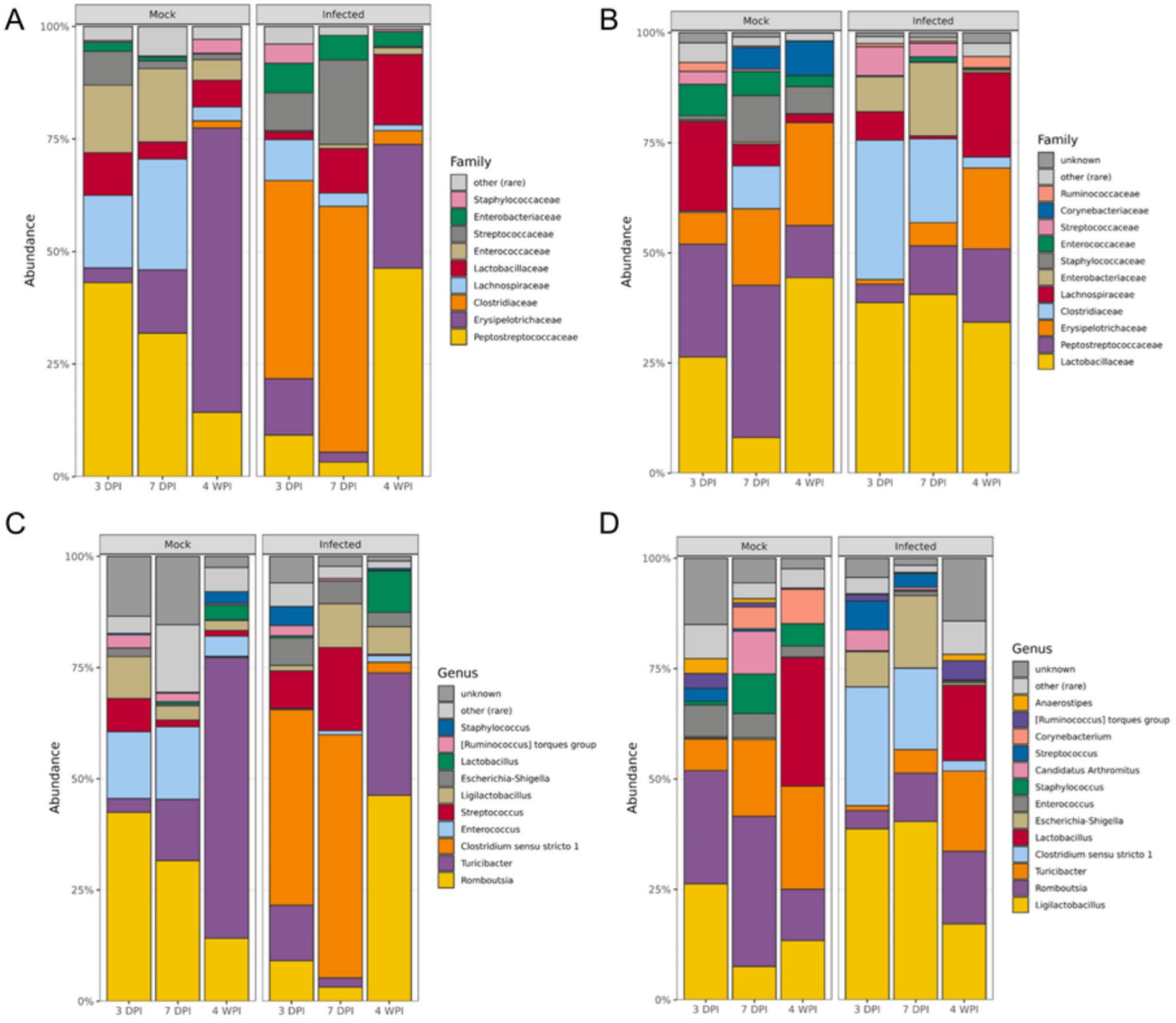

Fig. 7 Abundance of bacterial community in ileum among mock and inoculated groups. Abundance of ileal bacterial composition at the family level for 1WOA (**A**) and 3 WOA (**B**) inoculates among mock and infected groups. Abundance of ileal bacterial composition at the genus level for 1WOA (C) and 3 WOA (D) inoculates among mock and infected groups. DPI = days post infection. WPI= weeks post infection. WOA = weeks of age

Table 4 Experimental design

Age at infection (weeks)	Group	Inoculum	# Birds Euthanized			Total #Birds/Group
			3 dpi	7 dpi	4 WPI	
1	1 WOA	Medium	6	6	6	32
		TARV	6	6	6	32
3	3 WOA	Medium	6	6	6	32
		TARV	6	6	6	32
7	7 WOA	Medium	5	6	6	31
		TARV	6	6	6	32
Total			35	36	36	191

Statistical analysis

For viral titers, statistical differences were determined by One-way ANOVA followed by Tukey post hoc test for pair-wise comparisons. For histologic inflammation scores, and body weights, statistical analysis was determined using a Student's t test with Welch's correction. The data extrapolation was performed using GraphPad Prism 10.0.3.

Abbreviations

ASV	Amplicon sequence variants
CARV	Chicken arthritis reovirus
CPE	Cytopathic effect
DPI	Days post infection
ELISA	Enzyme linked immune sorbent assay
GE	Genomic equivalents
H & E	Hematoxylin and Eosin staining
IACUC	Institutional Animal Care and Use Committee
MCIC	Molecular and Cellular Imaging Center
PCoA	Principal coordinates analysis
PERMANOVA	PERmutational Multivariate ANalysis of VAriance
QT35	Quail fibrosarcoma cell line
RTqPCR	Quantitative reverse transcriptase polymerase chain reaction
SPF	Specific pathogen free
TARV	Turkey arthritis reovirus
TCID50	Median tissue culture infectious dose
WOA	Weeks of age
WPI	Weeks post infection

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04525-1.

Supplementary Material 1

Supplementary Material 2

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Author contributions

SK Wrote main manuscript, performed studies, data analysis, generated figures.JN Experimental design, performed studies, generated figures.PB Performed studiesKY Performed studiesAG Performed studiesMA Performed studiesCL Performed studiesJP Performed studies, trained student and assisted in bioinformaticsSG Performed studiesCWL Performed studies, experimental design, funding acquisitionGR Performed studies, experimental design, project oversightSPK Performed studies, experimental design, project oversight, editingAll authors reviewed the manuscript.

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Data availability

The 16 S fastq files will be uploaded in publicly available database. The codes and scripts will be made available via GitHub and link will be shared.

Declarations

Ethics approval and consent to participate

Animals were maintained, raised, and euthanized in accordance with ethical consideration set forth by The Ohio State University IACUC Animal Use Protocol #2011A000000109-R4. The animals for this study have been taken from the commercial farm in Ohio with the permission of the farm owner/authority. The informed consent obtained from the hatchery is attached and the hatchery remains anonymous.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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