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Protective Effect of a Hyperimmune Serum Against Homologous *Enterococcus cecorum* Infection in Experimentally Challenged Meat-Type Chickens

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Abstract: Knowledge about chickens' immune response to infection with *Enterococcus cecorum* (EC) and prophylactic strategies is scarce. This study aimed to investigate the protective effect of an EC-specific hyperimmune serum after experimental challenge with homologous EC. A total of 284 one-day-old meat-type chickens were randomly assigned to three groups (non-inoculated (C), passively immunized with hyperimmune serum and EC-inoculated (EPI), and EC-inoculated (E)). At 1 and 2 days post-hatch (dph), the hyperimmune serum was subcutaneously administered to each chicken in group EPI. Oral inoculation with EC was carried out at 2 dph. Blood samples and cloacal swabs were collected at 7, 14, 21, 28, and 35 dph and necropsy was carried out at 42/43 dph. Extraintestinal colonization with EC was significantly decreased in group EPI compared to group E. Additionally, femoral head necrosis and spondylitis of the free thoracic vertebra were significantly decreased in group EPI compared to group E. Flow cytometric analysis revealed a significant increase in heterophils, monocytes, and the heterophil/lymphocyte ratio in the peripheral blood of bacteriological positive chickens. Although the parenteral application of a hyperimmune serum lacks practicability in meat-type chicken flocks, the results of this study encourage further research on vaccination of meat-type chicken breeder flocks.

Keywords: *Enterococcus cecorum*; hyperimmune serum; meat-type chicken; passive immunization

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1. Introduction

Infections with pathogenic *Enterococcus cecorum* (EC) have emerged over the past decades [1–8]. EC is the causative agent of a disease often referred to as "enterococcal spondylitis" or "kinky back" [2,9]. The EC-associated disease causes increased mortality rates, therapy costs, and condemnation rates at slaughter. Thus, it results in reduced animal welfare and has a high economic impact [4,10,11]. During the first half of the growing period, affected chickens show clinical signs of general disease such as depression, ruffled feathers, and growth retardation. During necropsy, pericarditis, perihepatitis, and splenomegaly can be observed [4]. The predominant clinical sign in the second half of the fattening period is lameness, which often progresses to paresis of the affected chickens. Spondylitis of the free thoracic vertebra, which leads to compression of the spinal cord, and femoral head osteomyelitis can be observed during necropsy [6].

Nevertheless, knowledge about the chickens' immune response to infection with EC as well as therapeutic and prophylactic strategies against EC is severely limited. Although

metaphylactic antibiotic treatment was successful in a recently published study, it conflicts with the aim to reduce the use of antibiotics in poultry meat production [12]. A prophylactic approach is the administration of autogenous, polyvalent killed vaccines against EC in meat-type breeder chicken flocks. However, the composition of the vaccines and their application schedule are not standardized and therefore, the protective effect may differ between vaccines. This is supported by a study which revealed that maternal antibodies elicited by vaccination against EC did not protect the progeny against experimental challenge with EC [13]. A suitable model to investigate the influence of passively transferred antibodies is the administration of hyperimmune sera. Parenterally administered hyperimmune sera against different viruses and bacteria were successfully tested in experimental studies in chickens, ducks, and turkeys [14–21]. A common feature of all studies was the preparation of the hyperimmune serum in a homologous species. However, no such study has been conducted in regard to infections with EC in meat-type chickens yet.

Therefore, this study aimed to investigate the chickens' immune response to EC and the protective effect of passively transferred antibodies against EC. We hypothesized that infection with EC elicits a measurable humoral and cellular immune response. We further hypothesized that the parenteral administration of an EC-specific hyperimmune serum leads to a significant reduction of clinically affected chickens and inflammatory responses. Thus, the first objective was the preparation of an EC-specific hyperimmune serum. Subsequently, the second objective was to investigate the chickens' immune response and the hyperimmune serum's protective activity in a previously established in vivo model in meat-type chickens.

2. Materials and Methods

2.1. Hyperimmune Serum

2.1.1. EC-Specific Vaccine

A vaccine was prepared by an external manufacturer (RIPAC-LABOR GmbH, Potsdam, Germany). The inactivated pathogenic isolate EC 14/086/4/A was isolated from the heart of an affected meat-type chicken in a disease outbreak in 2014 [4]. It was used in a concentration of 10^7 colony forming units (CFU)/dose. A single dose was equal to 0.5 mL and aluminum hydroxide served as adjuvant.

2.1.2. Animal Experiment

Twenty female layer-type chickens were hatched from SPF eggs (VALO Biomedia GmbH, Osterholz-Scharmbek, Germany) and raised in the animal facility of the Clinic for Poultry, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. Chickens were kept in a floor pen on wood shavings under standard temperature conditions with feed (Deuka, Deutsche Tiernahrung Cremer GmbH & Co. KG, Dusseldorf, Germany) and water provided *ad libitum*. Perches, straw, and grains were provided as environmental enrichment. Light was switched on for the first 24 h post-hatch. Subsequently, the light program consisted of a 15 h light period and a 9 h dark period until the end of the experiment. Infrared lamps were provided for the first two days post-hatch (dph) and subsequently removed.

Each chicken was injected with 0.5 mL of the vaccine with a 21-gauge needle into the *musculus pectoralis* (pectoral muscle) at 70, 84, 98, 112, and 126 dph. Blood samples were collected from all animals at days of vaccination from the *vena basilica* (basilic vein). Additional blood samples were collected at 75, 80, and 91 dph. At 140 dph, all chickens were humanely sacrificed and the blood was collected for the preparation of the hyperimmune serum.

2.1.3. Preparation of the Hyperimmune Serum

Blood samples were centrifuged at 3000× *g* for 8 min (Heraeus Biofuge Haemo, Thermo Scientific, Thermo Fisher, Waltham, MA, USA) and sera collected at 140 dph were pooled. All serum samples were stored at −20 °C until further processing. To monitor seroconversion, individual serum samples from 70 to 126 dph were analyzed for EC-specific IgY using a recently established in-house ELISA [22]. Pooled sera from 140 dph were also analyzed for EC-specific IgY as well as the total amount of IgY using a commercially available indirect sandwich ELISA kit (Chicken IgG ELISA kit, Bethyl Laboratories, Fortis Life Sciences, Waltham, MA, USA).

2.2. Experimental Infection

2.2.1. Preliminary Study

The hyperimmune serum was administered subcutaneously into the *regio plicae lateralis* (knee crease) at one and two dph to confirm the successful absorption and systemic circulation of EC-specific antibodies. Briefly, 40 one-day-old meat-type chickens were obtained from a commercial hatchery (Brüterei Weser-Ems GmbH & Co. KG, Visbek, Germany) and randomly divided into two groups. All chickens were housed in floor pens on wood shavings under standard temperature conditions. Feed (Deuka, Deutsche Tiernahrung Cremer GmbH & Co. KG, Dusseldorf, Germany) and water were provided *ad libitum*. One group was inoculated with 0.3 mL of the EC-specific hyperimmune serum while the second group received 0.3 mL of an EC-negative control serum, which has previously been tested negative for EC-specific IgY using the in-house ELISA. At 2, 3, 4, and 5 days post-hatch, 5 chickens per group were humanely stunned with a percussive blow to the head and then instantly exsanguinated. Blood samples collected during bleeding of the chickens were centrifuged at 3000× *g* for 8 min and sera were analyzed for EC-specific IgY using the in-house ELISA.

2.2.2. Infection Study

All one-day-old Ross 308 chicks were obtained from a commercial hatchery (Brüterei Weser-Ems GmbH & Co. KG, Visbek, Germany). At the day of placement, 10 one-day-old chicks were examined for EC-specific IgY via ELISA and intestinal colonization with EC via real-time PCR. To fit the purpose of this study, various adjustments were made to the previously established EC-infection model [23]. Briefly, 284 one-day-old Ross 308 chickens were marked with individual wing tags and randomly assigned to three groups. Each group was kept in a separate floor pen on wood shavings under standard temperature conditions. Feed (Deuka, Deutsche Tiernahrung Cremer GmbH & Co. KG, Dusseldorf, Germany) and water were provided *ad libitum*. One group served as control (C), n = 94, one group was passively immunized and subsequently inoculated with EC (EPI), n = 95, and one group was inoculated with EC (E), n = 95. A total of 0.3 mL of the hyperimmune serum was administered subcutaneously at one and two dph into the *regio plicae lateralis* (knee crease). Oral inoculation with 0.5 mL of the inoculum (10^7 CFU of EC strain 14/086/4/A) or 0.5 mL physiological saline as mock was performed at two dph directly after the administration of the hyperimmune serum. Blood samples and cloacal swabs were collected at 7, 14, 21, 28, and 35 dph for further analysis. Blood samples were analyzed via ELISA and flow cytometry, while cloacal swabs were conducted to real-time PCR. Daily monitoring of all groups was performed to record clinical symptoms such as depression, ruffled feathers, lameness, and paresis. If a humane endpoint, which was determined in cooperation with the approving authority to ensure compliance with animal welfare regulations, was exceeded, the affected chicken was euthanized for welfare reasons and conducted to necropsy. The final necropsy was performed on 80 chickens (C and EPI) or 74 chickens

(E) per group at 42/43 dph. Half of each group was examined at 42 dph and the other half of each group at 43 dph. During necropsy, bodyweight, spleen weight, and gross lesions were documented. Blood samples were collected for further analysis via ELISA and flow cytometry. Amies medium swabs with charcoal (Hain Lifesciences GmbH, Nehren, Germany) were taken from heart, liver, spleen, free thoracic vertebra (FTV), and femoral heads for bacteriological examination for EC via culture. Dry swabs (Applimed SA, Châtel-St-Denis, Switzerland) were taken from the ceca for further analysis via real-time PCR for EC.

2.2.3. Inoculum

The pathogenic isolate EC 14/086/4/A was prepared for inoculation as previously published [23]. The optical density was determined using a Tecan Sunrise Reader (Tecan, Crailsheim, Germany). The initial suspension was diluted 1:10 with physiological saline to adjust the infectious dose to 2×10^7 CFU/mL and confirmed via total bacterial count determination using a tenfold dilution series. Physiological saline was used for mock inoculation of the control group.

2.2.4. DNA Isolation and Real-Time PCR

DNA was isolated from cloacal and cecal swabs using a commercially available kit (InnuPrep DNA Mini Kit 2.0, IST Innuscreen GmbH, Berlin, Germany) according to the manufacturer's instructions. Deviating from the protocol, samples and lysis buffer were incubated for 30 min instead of 10–15 min at 50 °C. Moreover, in the final step of the procedure, the DNA was eluted in 30 µL instead of the recommended 100–400 µL of DNase/RNase free water [12]. Real-time PCR to detect EC was carried out as previously published [12].

2.2.5. Bacteriological Examination

Bacteriological examination of extraintestinal tissues was carried out as previously published [12]. Amies medium swabs with charcoal (Hain Lifesciences GmbH) were plated on colistin-nalidixic acid (CNA) agar (Oxoid GmbH, Wesel, Germany) and incubated microaerophilically for 24 h at 37 °C. Small, grey, mucoid colonies with slight alpha hemolysis were subcultured and identified as EC. Then, 16S rRNA partial gene sequencing was performed to confirm the diagnosis (Microsynth AG, Lindau, Germany) [24–26].

2.2.6. EC-Specific ELISA

Blood samples were centrifuged at 3000× *g* for 8 min and serum samples were stored individually at −20 °C. Sera were analyzed in duplicates for EC-specific IgY using a previously established in-house ELISA [22].

2.2.7. Flow Cytometry

A no-lyse, no-wash, single-step one-tube procedure was used for flow cytometry analysis [27]. Briefly, fresh EDTA tripotassium blood samples were diluted 1:500 in PBS (AppliChem GmbH, Darmstadt, Germany) supplemented with 1% bovine serum albumin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and 0.4% EDTA (Sigma-Aldrich, St. Louis, MO, USA), pH 7.4. An antibody cocktail was prepared using the antibodies listed in Table S1. Final antibody concentrations and a compensation matrix were determined prior to this study. Round-bottom 96-well plates (Nunc, Wiesbaden, Germany) were prepared with 5 µL of the antibody cocktail per well. Subsequently, 235 µL of the diluted EDTA–blood samples was added per well and incubated for at least two hours protected from light and on ice. After the incubation period, 30 µL per well was measured using the MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany).

Immediately prior to the measurement, 3 µL 7-AAD (Miltenyi Biotec B.V. & Co. KG) was added to each well as a marker for cell viability. Files were analyzed using FlowLogic 7.3 (Inivai Technologies, Mentone, Australia). Doublets and dead cells were excluded from the analysis. For gating of the cell populations, samples were grouped per time point. The final gating strategy is displayed in Figure S1.

Due to a technical error of the flow cytometer, statistical analysis of samples at 21 dph was not possible. At 35 dph, 5 samples (group C), 3 samples (group EPI), and 6 samples (group E) were not analyzed due to clotting.

2.3. Statistical Analysis

Statistical analysis was carried out using SAS Enterprise Guide (Version 7.15, SAS Institute Inc., Cary, NC, USA). Graphs were created using GraphPad Prism (Version 6.02, GraphPad Software, LLC, San Diego, CA, USA).

Descriptive statistics were used to analyze clinical signs and gross lesions. The Kruskal–Wallis test and Wilcoxon's rank-sum test were used to evaluate significant differences in flow cytometry results, bodyweight, and spleen/bodyweight ratios between groups. Fisher's least significant difference test was used to evaluate differences in flow cytometry results and S/P ratios between bacteriological positive and negative chickens. Fisher's exact test was used to analyze the proportion of EC-positive chickens via culture and via ELISA as well as the detection rates of EC-DNA via real-time PCR. Where applicable, the Benjamini–Hochberg correction method was applied to adjust *p*-values for multiple testing [28]. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Hyperimmune Serum

EC-specific antibodies were first detected at 80 dph (10 days post-prime vaccination). S/P ratios steadily increased until the end of the study at 140 dph (Figure S2). The total IgY concentration of the final hyperimmune serum was 14 mg/mL.

3.2. Bodyweight

Final bodyweights were documented for all meat-type chickens at 42/43 dph (Table 1). Mean bodyweight was lowest in group E, but no significant differences were observed between groups ($p > 0.05$).

Table 1. Clinical symptoms, performance parameters, and pathological findings after inoculation with EC.

	Impaired General Condition	Lameness to Paresis	Bodyweight (g)	Spleen/Bodyweight Ratio (g/kg)	Pericarditis	Perihepatitis	Spondylitis of the FTV	Femoral Head Osteomyelitis
C	2/85 (2.4%) ^{a}	2/85 (2.4%) ^{a}	3327 ± 383 ^{ns}	0.928 ± 0.24 ^{a}	4/85 (4.7%) ^{a}	0/85 (0%) ^{ns}	0/82 (0%) ^{a}	0/82 (0%) ^{a}
EPI	8/89 (9%) ^{a}	9/89 (10.1%) ^{ab}	3262 ± 465 ^{ns}	1.049 ± 0.26 ^{b}	12/89 (13.5%) ^{a}	4/89 (4.5%) ^{ns}	8/85 (9.4%) ^{b}	2/85 (2.4%) ^{a}
E	27/95 (28.4%) ^{b}	18/95 (19%) ^{b}	3191 ± 501 ^{ns}	1.105 ± 0.39 ^{b}	39/95 (41.1%) ^{b}	6/95 (6.3%) ^{ns}	23/87 (26.4%) ^{c}	12/87 (13.8%) ^{b}

Number of chickens with clinical signs and pathological findings throughout the study. Bodyweight and spleen/bodyweight ratio were determined at 42/43 dph. Impaired general condition was indicated by depression, ruffled feathers, and closed eyes. Fisher's exact test was used to compare clinical signs and pathological findings between groups. Groupwise comparisons of bodyweight and spleen/bodyweight ratio were performed using the Kruskal–Wallis test and Wilcoxon's rank-sum test. Bodyweight is presented as mean ± SD. Where applicable, *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction method. Different superscript letters indicate significant differences between groups. C = control, E = EC-inoculated, EC = *Enterococcus cecorum*, EPI = EC-inoculated, passively immunized, FTV = free thoracic vertebra, ns = not significant.

3.3. Clinical Signs and Pathological Findings

Clinical symptoms and pathological findings of the EC-associated disease were reproduced in groups EPI and E (Table 1). Throughout the study, significantly more chickens

exhibited symptoms of disease such as depression, ruffled feathers, and growth retardation in group E compared to group C and EPI ($p < 0.05$). Furthermore, significantly more lame chickens were observed in group E compared to group C ($p < 0.05$), whereas lameness was detected in a similar number of chickens in group E and EPI ($p > 0.05$). Impaired general condition and lameness in group C were attributed to individual cases of splayed legs.

The detection rate of spondylitis differed significantly between all groups with the highest number of affected chickens observed in group E ($p < 0.05$). Femoral head osteomyelitis and pericarditis were detected significantly more often in group E compared to either group C or EPI ($p < 0.05$). In addition to that, perihepatitis was observed in both group EPI and E, but the number of affected chickens was comparable in both groups ($p > 0.05$).

Spleen/bodyweight ratios were calculated for all chickens at 42/43 dph (Table 1). Spleen/bodyweight ratios were significantly higher in group EPI and E compared to group C ($p < 0.05$).

3.4. Intestinal Colonization with EC

Chicks at day of placement tested negative via real-time PCR. Results from real-time PCR analysis of cloacal/cecal swabs are displayed in Figure 1. At seven dph, 100% of the cloacal swabs from inoculated chickens in groups EPI and E were tested positive for EC via real-time PCR, whereas all cloacal swabs from group C were classified as negative ($p < 0.05$). Similar results were observed at 14, 21, 28, and 35 dph with more than 90% positive samples in groups EPI and E and 3/82 (3.6%) or less positive samples in group C ($p < 0.05$). At the end of the study, a reduced number of the cecal swabs was included in the analysis due to inhibition of the samples. Analysis of cecal swabs revealed similar results to previous time points in groups C and EPI. However, the number of EC-positive samples in group E (31/68, 45.6%) dropped significantly in comparison to group EPI (66/68, 97%, $p < 0.05$).

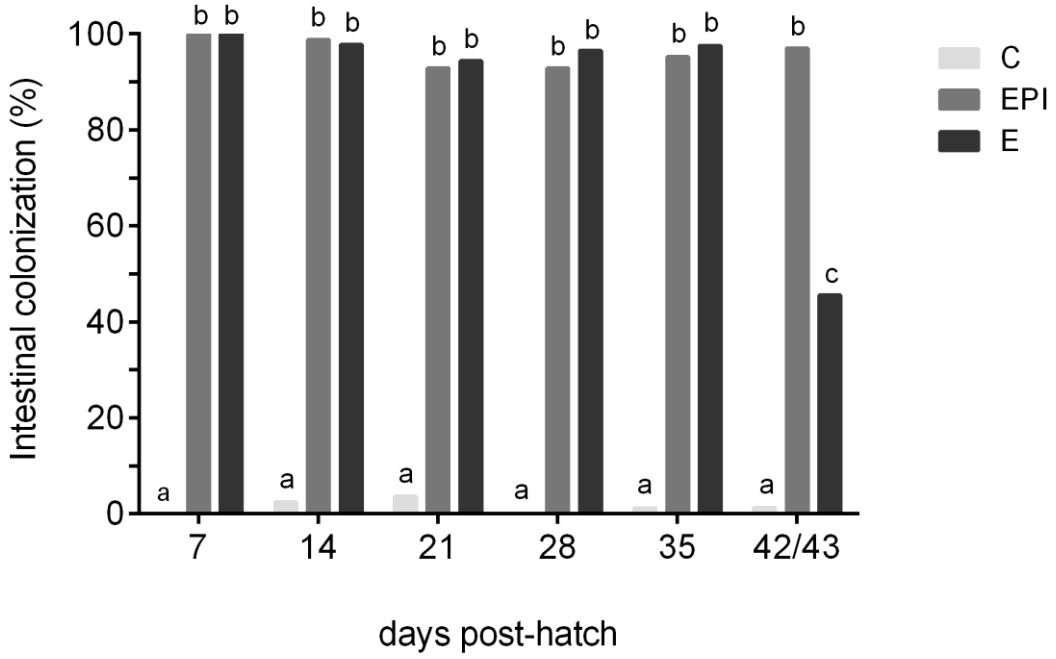

Figure 1. Intestinal colonization with EC. Proportion of intestinal colonization with EC throughout the study. Cloacal swabs were collected at 7, 14, 21, 28, and 35 dph. Cecal swabs were collected at 42/43 dph. All samples were subjected to DNA isolation and subsequent real-time PCR. Ct values below 36 were considered positive. Fisher's exact test was used to compare groups per time point. *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction

method. Different superscript letters indicate significant differences between groups. At 7 dph: n = 84 (C), 89 (EPI), 92 (E); 14 dph: n = 83 (C), 87 (EPI), 90 (E); 21 dph: n = 82 (C), 85 (EPI), 89 (E); 28 dph: n = 82 (C), 85 (EPI), 87 (E); 35 dph: n = 81 (C), 84 (EPI), 83 (E); 42/43 dph: n = 73 (C), 68 (EPI and E). C = control, dph = days post-hatch, E = EC-inoculated, EC = *Enterococcus cecorum*, EPI = EC-inoculated, passively immunized.

3.5. Extraintestinal Colonization with EC

Extraintestinal colonization was determined for all chickens at 42/43 dph and for all chickens that had to be euthanized prior to the end of the experiment. Throughout the study, EC was detected in 21/89 (23.6%) of the chickens in group EPI and 37/93 (39.8%) of the chickens in group E via bacteriological examination (Figure 2). Chickens from group C remained negative throughout the entire study. EC was detected significantly more often in group E compared to the other groups ($p < 0.05$). Significant differences were further observed between group C and EPI ($p < 0.05$).

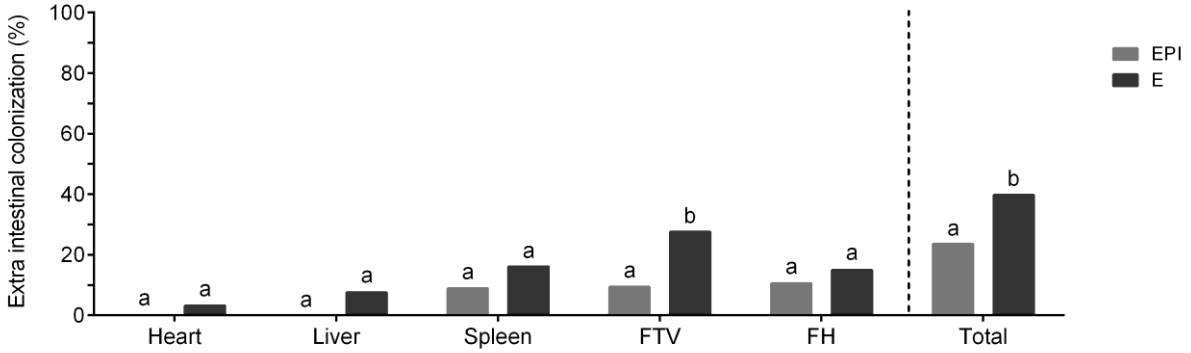

Figure 2. Extraintestinal colonization with EC. Proportion of EC-positive samples sorted by organ and total proportion of EC-positive chickens per group. Swabs from heart, liver, spleen, FTV, and FHs were examined for EC via culture. Samples from group C were tested negative throughout the study. Fisher's exact test was used to compare groups. *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction method. Different superscript letters indicate significant differences between groups. n = 85 (C), 89 (EPI), 95 (E). C = control, E = EC-inoculated, EC = *Enterococcus cecorum*, EPI = EC-inoculated, passively immunized, FTV = free thoracic vertebra, FHs = femoral heads.

Organs from which EC was reisolated are displayed in Figure 2. EC was most frequently isolated from the free thoracic vertebra in groups E and EPI with the highest detection rate in group E compared to groups C and EPI ($p < 0.05$). In addition, there was a trend towards more positive femoral heads and spleens in group E compared to group EPI, but the numbers did not differ significantly ($p > 0.05$). In group E, EC was also isolated from the heart and liver, but no significant differences were observed compared to either group C or EPI ($p > 0.05$). Overall, extraintestinal colonization of multiple organs was observed significantly more often in group E (20/93, 21.5%) compared to group EPI (5/89, 5.6%, $p < 0.05$).

3.6. Detection of Passively Transferred EC-Specific Antibodies and Seroconversion After Inoculation with EC

Chicks at day of placement tested negative for EC-specific IgY. Proportions of EC-positive chickens via ELISA are displayed in Table 2. Seroconversion after subcutaneous inoculation with EC-specific antibodies was confirmed at 2, 3, and 4 dph. After experimental infection, seroconversion was first observed in group E at 21 dph, whereas EC-specific IgY was first detected at 28 dph in group EPI. At the end of the study, seroconversion was observed in 51/74 (68.9%) in group E and in 26/80 (32.5%) in group EPI. Significant differences were observed between all groups at 28, 35, and 42/43 dph ($p < 0.05$). Irrespective of

group, S/P ratios were significantly higher in bacteriological positive chickens compared to bacteriological negative chickens from 21 dph onwards ($p < 0.05$, Figure S3D). Serum samples from group C remained negative throughout the study.

Table 2. Seroconversion after subcutaneous inoculation with EC-specific antibodies and oral inoculation with EC.

		Number of Chickens Positive for EC-Specific Antibodies at Sampling Time Points									
		2 dph	**3 dph**	**4 dph**	**5 dph**	**7 dph**	**14 dph**	**21 dph**	**28 dph**	**35 dph**	**42 dph**
Preliminary study	EC-specific hyperimmune serum	3/5	4/5	2/5	0/5	nd	nd	nd	nd	nd	nd
	EC-antibody negative serum	1/5	0/5	1/5	1/5	nd	nd	nd	nd	nd	nd
Infection study	EC-specific hyperimmune serum, EC-inoculated (EPI)	nd	nd	nd	nd	0/91 (0%)	0/90 (0%)	0/85 (0%) [a]	9/85 (10.6%) [a]	25/84 (29.8%) [a]	26/80 (32.5%) [a]
	EC-inoculated (E)	nd	nd	nd	nd	0/89 (0%)	0/87 (0%)	10/89 (11.2%) [b]	46/87 (52.9%) [b]	53/84 (63.1%) [b]	51/74 (68.9%) [b]

Proportion of EC-positive serum samples via ELISA. In the preliminary study, one group was inoculated with an EC-specific hyperimmune serum at 1 and 2 dph while the other group received an EC-antibody negative control serum. Serum samples were collected at 2, 3, 4, and 5 dph. In the infection study, group EPI received an EC-specific hyperimmune serum at 1 and 2 dph. Both group EPI and E were orally inoculated with EC at 2 dph. Serum samples were collected at 7, 14, 21, 28, 35, and 42/43 dph. All samples were analyzed with an EC-specific ELISA. Seroconversion was first observed at 21 dph. Samples from the control group (C) remained negative throughout the study. The negative–positive cut-off value was calculated as the mean S/P ratio of the negative control + 2 × SD and was equal to 0.15. S/P ratios ≥ 0.15 were considered positive. Fisher's exact test was used to compare groups per time point. *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction method. Different superscript letters indicate significant differences between groups. C = control, dph = days post-hatch, E = EC-inoculated, EC = *Enterococcus cecorum*, EPI = EC-inoculated, passively immunized, nd = not done, S/P ratio = sample/positive ratio.

3.7. Cellular Immune Response After Inoculation with EC

Changes in peripheral heterophil and monocyte concentrations were observed both with increasing age and due to infection with EC. At all time points, concentrations of peripheral heterophils were significantly increased in group E compared to the other groups and in group EPI compared to group C ($p < 0.05$, Figure 3A). In general, comparison of bacteriological positive and negative chickens revealed that heterophil concentrations were significantly higher in bacteriological positive chickens at all time points ($p < 0.05$, Figure S3C). Concentrations of peripheral monocytes were significantly increased at 14, 28, and 35 dph in group E and at 14 and 28 dph in group EPI ($p < 0.05$, Figure 3B). Additionally, monocyte concentrations were significantly increased in bacteriological positive chickens irrespective of group compared to bacteriological negative chickens from 14 dph onwards ($p < 0.05$, Figure S3B).

Independent of group, the heterophil/lymphocyte (H/L) ratio was significantly increased in bacteriological positive chickens compared to bacteriological negative chickens throughout the study ($p < 0.05$, Figure S3A). Groupwise comparisons revealed that the H/L ratio was significantly increased in group E compared to group C at all time points ($p < 0.05$, Figure 4). Additionally, significant differences between groups E and EPI were observed at 14, 28, and 42/43 dph ($p < 0.05$, Figure 4).

CD4+ and CD8+ lymphocyte concentrations increased with age, whereas only a slight increase was observed due to infection with EC. While CD8+ lymphocyte concentrations were significantly higher in group E at 7 and 14 dph, CD4+ lymphocyte concentrations were significantly increased at 28 and 35 dph ($p < 0.05$, Figure S4A,B). Thrombocyte concentrations were lowest at 7 dph and did not differ significantly between groups from 14 dph onwards ($p > 0.05$, Figure S4C).

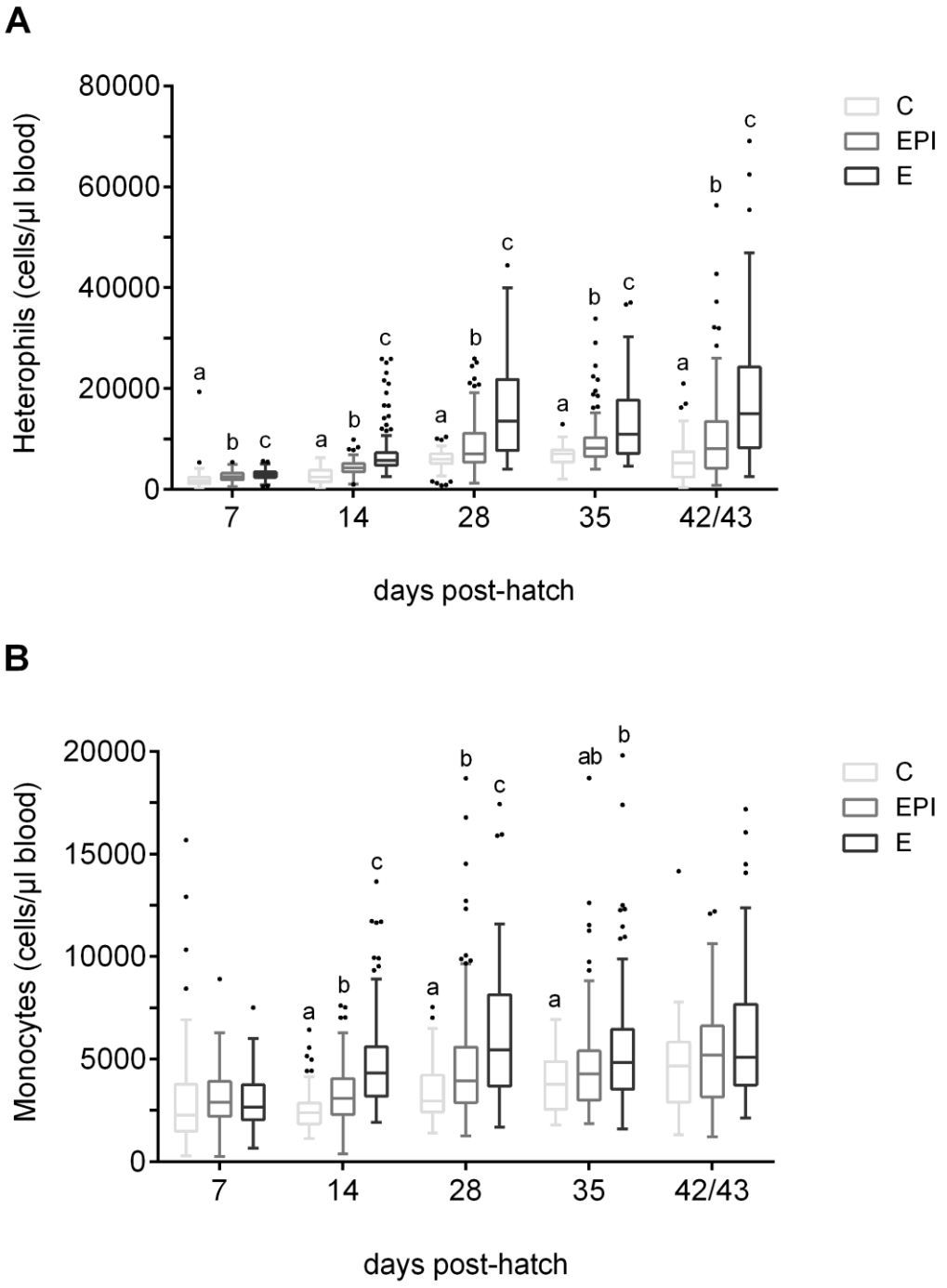

Figure 3. Inflammatory response after inoculation with EC. Concentrations of peripheral heterophils (**A**) and monocytes (**B**) determined via flow cytometry of whole blood samples at 7, 14, 28, 35, and 42/43 dph. Boxes represent the 25th to 75th percentile. Whiskers cover 1.5 × the interquartile range. Outliers are presented as black dots. Groupwise comparisons were performed using the Kruskal–Wallis test and Wilcoxon's rank-sum test. *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction method. Different superscript letters indicate significant differences between groups. At 7 dph: n = 82 (C), 88 (EPI), 92 (E); 14 dph: n = 83 (C), 86 (EPI), 90 (E); 28 dph: n = 82 (C), 85 (EPI), 86 (E); 35 dph: n = 76 (C), 82 (EPI), 78 (E); 42/43 dph: n = 80 (C and EPI), 74 (E). C = control, dph = days post-hatch, E = EC-inoculated, EC = *Enterococcus cecorum*, EPI = EC-inoculated, passively immunized.

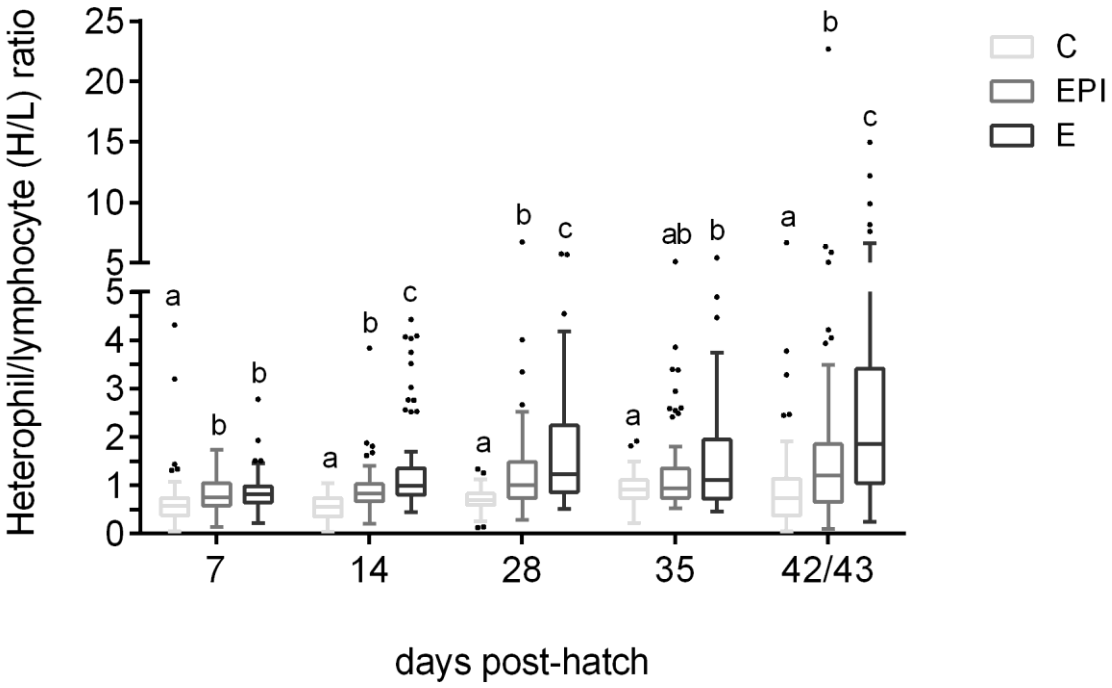

Figure 4. Heterophil/lymphocyte (H/L) ratios after inoculation with EC. H/L ratios were calculated from flow cytometry data at 7, 14, 28, 35, and 42/43 dph. Boxes represent the 25th to 75th percentile. Whiskers cover 1.5 × the interquartile range. Outliers are presented as black dots. Groupwise comparisons were performed using the Kruskal–Wallis test and Wilcoxon's rank-sum test. *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction method. Different superscript letters indicate significant differences between groups. At 7 dph: n = 82 (C), 88 (EPI), 92 (E); 14 dph: n = 83 (C), 86 (EPI), 90 (E); 28 dph: n = 82 (C), 85 (EPI), 86 (E); 35 dph: n = 76 (C), 82 (EPI), 78 (E); 42/43 dph: n = 80 (C and EPI), 74 (E). C = control, dph = days post-hatch, E = EC-inoculated, EC = *Enterococcus cecorum*, EPI = EC-inoculated, passively immunized.

4. Discussion

Infection with pathogenic EC remains a major problem in modern meat-type chicken husbandry. However, few data are available regarding the chickens' immune response to infection with EC and immune protection against EC. Therefore, it was the aim of this study to investigate the chickens' immune response to infection with EC and the protective effect of passively transferred, EC-specific antibodies.

4.1. Absorption of the Hyperimmune Serum

The biological half-live of IgY is known to be 3–4 days [29,30]. At 7 dph, EC-specific antibodies were not detected in any of the groups. However, monitoring of EC-specific antibodies at a closer interval revealed that sera reacted positive via ELISA at 2, 3, and 4 dph. Therefore, the absence of EC-specific antibodies at 7 dph in group EPI is most likely due to short half-life and fast degradation. Differences in colonization and immune response between groups E and EPI in this study may be attributed to the hyperimmune serum.

4.2. EC-Specific Antibodies Control Extraintestinal Colonization with EC

The clinical disease was successfully reproduced in group E and EPI. Higher isolation rates compared to previous infection studies conducted in our institution are most likely attributed to the different study design with a single necropsy time point at the end of this study [12,31]. Comparison of individual groups revealed that target bodyweight was met independent of treatment or infection status [32]. This stands in contrast to field reports, where a reduced bodyweight gain has been observed in EC-affected flocks [33].

The subcutaneous administration of EC-specific IgY led to a delayed onset of seroconversion and extraintestinal colonization with EC. Additionally, the inflammatory response measured by peripheral blood leukocytes (PBL) was significantly reduced in group EPI compared to group E. These results suggest that circulating EC-specific IgY may shorten the period in which EC translocates from the intestine and colonizes extraintestinal organs, thereby leading to a reduced inflammatory response and seroconversion. Therefore, these results may provide a promising basis for the optimization of currently existing vaccination regimens in meat-type breeder chickens to confer sufficient protection of the progeny by maternal antibodies.

4.3. Increase in Innate Leukocytes After Inoculation with EC

Extensive literature research in commonly used scientific databases (Google, Scopus, CAB Direct, and PubMed) revealed a lack of knowledge about the inflammatory response after inoculation with EC. To our knowledge, this is the first report of white blood cell counts after infection with EC in meat-type chickens. White blood cell counts in group C were comparable to previously published values [27,34]. In groups EPI and E, increased concentrations of heterophils and monocytes representing the innate immune response were observed. This is in agreement with studies on *Salmonella enterica* serovar Enteritidis, *Histomonas meleagridis*, and *Erysipelothrix rhusiopathiae* [35–37]. Previous studies have shown infiltration of heterophils and macrophages at the site of inflammation, which corresponds with our findings in the blood [3,4,38]. Lower concentrations of heterophils and monocytes in group EPI compared to group E suggest that the hyperimmune serum may have reduced the circulating bacterial load, thereby mitigating the innate immune response. Interestingly, an in vitro study has shown that pathogenic strains of EC may be able to avoid opsonophagocytosis of macrophages [13]. However, it is not known whether the same is true for heterophils. Future studies utilizing fluorescence-labeled EC may contribute to determine the association of EC to heterophils and monocytes in the peripheral blood. Since both cell types were elevated throughout the study, it may also be interesting to investigate whether EC-inoculated chickens show bacteremia throughout the entire production cycle. This is of particular interest because the EC-associated disease is mainly associated with local sites of inflammation such as the FTV and the femoral heads. Interestingly, the chronic inflammatory response did not have a negative impact on bodyweight in this study. However, elevated H/L ratios in bacteriological positive chickens at all time points suggest that infection with EC elicits stress in affected chickens, which is in agreement with studies conducted with *Salmonella enterica* serovar Enteritidis [39].

4.4. Practical Relevance

This study used the application of an EC-specific hyperimmune serum as a model to investigate the effect of circulating IgY on infection with EC, which can be achieved by vaccination of breeder chickens. Vaccination of breeder chickens is a promising approach to confer protection of the progeny against pathogens. After deposition of maternal antibodies into the egg, they are subsequently absorbed by the embryo [40]. While IgY is primarily transferred from the egg yolk to the serum, IgA and IgM are transferred to the embryo's gastrointestinal tract via the albumen [41,42]. Vaccination of breeder chickens focuses on the transfer of maternal IgY into the progeny's circulation, which has a close resemblance to the experimental design of this study. Hence, the results may be applied to the mode of action of breeder chicken vaccination. Since no licensed vaccines against EC are available, autogenous vaccines are commonly used [43,44]. As a result, dose, number of applications, and immunogenicity may differ between flocks. Interestingly, a study from the US has shown that vaccination of breeder chickens with a polyvalent killed vaccine did not protect

their progeny against the EC-associated disease [13]. A variety of factors may be responsible for the different results observed in the current study and the one from the US. To begin with, study set-ups differed between both studies. The higher number of booster vaccinations in the current study may have led to increased protection compared to the study investigating maternally derived antibodies. However, higher numbers of booster vaccination are less feasible for veterinarians on farm. Furthermore, the composition of the vaccine, dose, and the strain used for the subsequent challenge of one-day-old chicks differed between the studies. This leaves a wide range of options to adjust the generation of EC-specific IgY. On the side of the vaccine, strain, adjuvant, and number of doses may need to be adjusted to elicit consistently high levels of EC-specific IgY. The current study has shown that a homologous vaccine had a significant impact on the course of the EC-associated disease, which supports the use of autogenous vaccines. Future studies on the antigenic determinants of EC may help to increase the vaccines' efficacy and elicit higher levels of EC-specific antibodies.

5. Conclusions

In conclusion, oral inoculation with EC elicited a measurable humoral and cellular immune response in meat-type chickens. The subcutaneous administration of homologous, EC-specific antibodies provided partial protection against the EC-associated disease. Although the disease was not prevented entirely, the onset of clinical symptoms and extraintestinal colonization was delayed and the proportion of affected chickens significantly reduced. However, adjustment of current prophylactic strategies based on a more profound understanding of the pathogenesis is necessary to successfully combat the EC-associated disease. This may be a multidirectional approach based on passive immunization but also including other preventive strategies such as increased biosecurity or the use of less susceptible chickens. The subcutaneous administration of a hyperimmune serum is not practicable in large-scale operations, but the results of this study encourage further research to improve currently existing autogenous vaccines and to develop new vaccines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/poultry4010008/s1, Table S1: Antibodies used for flow cytometric analysis; Figure S1: Gating strategy. Discrimination of thrombocytes, monocytes, heterophils, CD8+ T-cells, and CD4+ T-cells. EDTA-blood was stained with anti-CD45-FITC, Kul01-FITC, anti-CD51/61-PE, anti-CD4-PE-Cy7, and anti-CD8α-APC and analyzed via flow cytometry. Dead cells were excluded based on 7-AAD staining. The displayed gating strategy was used to identify thrombocytes (I), monocytes (II), heterophils (III), CD8+ T-cells (IV), and CD4+ T-cells (V) based on marker expression (A,B) and subsequent FSC/SSC characteristics (C–G); Figure S2: S/P ratios hyperimmune serum. Development of EC-specific antibodies after vaccination with an inactivated, EC-specific vaccine. Time points of vaccination are indicated by the syringe below the time point. S/P ratios are presented as boxplots per time point. Boxes represent the 25th to 75th percentile. Whiskers extend from minimum to maximum values. All samples were analyzed with an EC-specific in-house ELISA. The negative–positive cut-off value (dotted line) was calculated as the mean S/P ratio of the negative control + 2 $\times$ SD and was equal to 0.15. S/P ratios $\geq$ 0.15 were considered positive. n = 20 per time point. dph = days post-hatch; Figure S3: Comparison of H/L ratios (A), monocytes (B), heterophils (C), and S/P ratios (D) of bacteriological negative and positive chickens. Blood samples were collected at 7, 14, 21, 28, 35, and 42/43 dph and conducted to analysis via flow cytometry (A–C) or an EC-specific in-house ELISA (D). The negative–positive cut-off value was calculated as the mean S/P ratio of the negative control + 2 $\times$ SD and was equal to 0.15. S/P ratios $\geq$ 0.15 were considered positive. Fisher's least significant difference test was used to evaluate differences in flow cytometry results and S/P ratios between bacteriological positive and negative chickens independent of group. n = 40–58 (bacteriological positive), 184–205 (bacteriological negative). Figure S4: CD8+ lymphocyte,

CD4+ lymphocyte, and thrombocyte concentrations after inoculation with EC. Concentrations of peripheral CD8+ lymphocytes (A), CD4+ lymphocytes (B), and thrombocytes (C) were determined via flow cytometry of whole blood samples at 7, 14, 28, 35, and 42/43 dph. Boxes represent the 25th to 75th percentile. Whiskers cover 1.5 × the interquartile range. Outliers are presented as black dots. Groupwise comparisons were performed using the Kruskal–Wallis test and Wilcoxon's rank-sum test. *p*-value adjustments for multiple testing were performed using the Benjamini–Hochberg correction method. Different superscript letters indicate significant differences between groups. At 7 dph: n = 82 (C), 88 (EPI), 92 (E); 14 dph: n = 83 (C), 86 (EPI), 90 (E); 28 dph: n = 82 (C), 85 (EPI), 86 (E); 35 dph: n = 76 (C), 82 (EPI), 78 (E); 42/43 dph: n = 80 (C and EPI), 74 (E). C = control, dph = days post-hatch, E = EC-inoculated, EC = Enterococcus cecorum, EPI = EC-inoculated, passively immunized.

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Institutional Review Board Statement: The study design was approved by the Animal Ethics Committee of the University of Veterinary Medicine Hannover, Foundation, Hannover, Germany (TVG-2023-P-19) and conducted in accordance with EU directive 2010/63/EU and the regulations on animal welfare and animal experimentation in Germany (33.8-42502-04-20/3551 approved on 4 June 2021, 33.8-42502-04-23-00306 approved on 14 April 2023).

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