Vaccine 41 (2023) 1342–1353

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Aerosol delivered irradiated *Escherichia coli* confers serotype-independent protection and prevents colibacillosis in young chickens

Surya Paudel [a,b,*,1], Claudia Hess [a], Mohamed Kamal Abdelhamid [a], Manolis Lyrakis [c], Viskam Wijewardana [d], Richard Thiga Kangethe [d], Giovanni Cattoli [d], Michael Hess [a]

[a] Clinic for Poultry and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria
[b] Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong
[c] Platform for Bioinformatics and Biostatistics, Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Vienna, Austria
[d] Animal Production and Health Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency IAEA, Vienna, Austria

ARTICLE INFO

Article history:
Received 29 August 2022
Received in revised form 30 October 2022
Accepted 3 December 2022
Available online 13 January 2023

Keywords:
Avian pathogenic *Escherichia coli*
Colibacillosis
Vaccination
Irradiation
Chickens

ABSTRACT

Escherichia coli causes colibacillosis in chickens, which has severe economic and public health consequences. For the first time, we investigated the efficacy of gamma-irradiated *E. coli* to prevent colibacillosis in chickens considering different strains and application routes. Electron microscopy, alamarBlue assay and matrix assisted laser desorption/ionization time-of- flight mass spectrometry showed that the cellular structure, metabolic activity and protein profiles of irradiated and non-treated *E. coli* PA14/17480/5-ovary (serotype O1:K1) were similar. Subsequently, three animal trials were performed using the irradiated *E. coli* and clinical signs, pathological lesions and bacterial colonization in systemic organs were assessed.

In the first animal trial, the irradiated *E. coli* PA14/17480/5-ovary administered at 7 and 21 days of age via aerosol and oculonasal routes, respectively, prevented the occurrence of lesions and systemic bacterial spread after homologous challenge, as efficient as live infection or formalin-killed cells. In the second trial, a single aerosol application of the same irradiated strain in one-day old chickens was efficacious against challenges with a homologous or a heterologous strain (undefined serotype). The aerosol application elicited better protection as compared to oculonasal route. Finally, in the third trial, efficacy against *E. coli* PA15/19103–3 (serotype O78:K80) was shown. Additionally, previous results of homologous protection were reconfirmed. The irradiated PA15/19103–3 strain, which also showed lower metabolic activity, was less preferred even for the homologous protection, underlining the importance of the vaccine strain. In all the trials, the irradiated *E. coli* did not provoke antibody response indicating the importance of innate or cell mediated immunity for protection.

In conclusion, this proof-of-concept study showed that the non-adjuvanted single aerosol application of irradiated "killed but metabolically active" *E. coli* provided promising results to prevent colibacillosis in chickens at an early stage of life. The findings open new avenues for vaccine production with *E. coli* in chickens using irradiation technology.

1. Introduction

Extraintestinal diseases in poultry caused by *Escherichia coli,* a gram-negative bacillus in the family Enterobacteriaceae are called colibacillosis [1]. It affects all age groups of chickens, mainly causing omphalitis, airsacculitis, pericarditis, perihepatitis, peritonitis, salpingitis, cellulitis and femoral head necrosis. High economic losses due to mortality and reduced performance as well as growing antimicrobial resistance in *E. coli* are the major concerns associated with the disease [1,2].

Increasing demand to reduce the antibiotic use in livestock has emphasized for the effective vaccine development to prevent colibacillosis in poultry, and numerous efforts were made in the past [1,3–6]. Due to the complex genetic traits and heterogenicity of *E. coli* isolates, concerns regarding the strain coverage and feasibility of application still exist and the number of licensed products is

* Corresponding author at: Clinic for Poultry and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria.
E-mail address: spaudel@cityu.edu.hk (S. Paudel).
[1] Current address: Department of Infectious Diseases and Public Health, Jockey Club College of Veterianry Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong

https://doi.org/10.1016/j.vaccine.2022.12.002
0264-410X/

very low. In a field study, vaccination of breeders with a commercial inactivated vaccine (Nobilis® E. coli Inac., MSD Animal health) led to lower incidences of colibacillosis in parents but had no influence on the early week mortality, average body weight and feed conversion ratio of offspring [7]. Autogenous vaccines are used in recent years but conflicting results were reported with regards to their efficacy [8–10]. Live or live attenuated vaccines were produced by using non-pathogenic strains [11] or knockouts of essential genes, for instance *aro*A or *crp* deleted mutants (Poulvac® E. coli, Zoetis; Nisseiken Avian Colibacillosis Vaccine® "CBL, Nisseiken Co., ltd.). The *aro*A deleted Poulvac® E. coli vaccine protected birds against experimental homologous and heterologous challenges [12] and reduced the overall mortality and colibacillosis-associated lesions in a field trial [13]. In contrast, birds vaccinated only with this vaccine were not protected against experimental challenges [14–16] and the efficacy was only enhanced when vaccinated pullets were boosted with an autogenous vaccine [16]. In a different study, *aro*A deletion mutant of another O78 *E. coli* strain was not efficacious against *E. coli*-O2 challenge, thus the protection was serogroup specific [17]. Likewise, a *crp* deleted O78 *E. coli* vaccine was effective against the same serotype [18,19] but not against a challenge with *E. coli*-O125 [18]. Thus, expanding the portfolio of *E. coli* vaccine in chickens considering feasibility of application in the field is a necessity in poultry, which was also addressed by the OIE [20].

The irradiation technologies using radioactive isotopes, electron beam or X-ray were used to generate vaccines against numerous bacterial, viral and protozoal pathogens in humans and animals [21]. After irradiation, microbial cells retain functional antigenic expression and metabolic activity but fail to multiply primarily due to damage in nucleic acids [22]. Previous works showed that irradiated bacteria are more efficient in stimulating host immune response, predominantly *T*-cell response compared to heat-or formalin killed bacterins [23–26]. Thus, employing the same technique, the present study investigated the efficacy of a gamma-irradiated *E. coli* as a novel prophylactic measure for the control of colibacillosis in chickens considering different routes, strains and age of vaccination.

2. Materials and methods

2.1. Bacterial isolates

E. coli PA14/17480/5-ovary (serotype O1:K1) was isolated from the ovary of a chicken with colibacillosis [27] and characterized *in vitro* [28]. For the challenge in trial 1, the *lux*-tagged *E. coli* PA14/17480/5-ovary was used [29]. *E. coli* PA16/13200-animal2/clone3 was isolated from the liver of a colibacillosis-affected layer and was not typeable using antibodies against O1:K1, O2:K1 or O78:K80 [27]. *E. coli* PA15/19103–3 (serotype O78:K80) was isolated from femur of a broiler with femoral head necrosis [30].

2.2. Preparation of irradiated bacteria suspensions

E. coli PA14/17480/5-ovary and PA15/19103–3 were processed for irradiation based on the protocols explained previously [26]. Details of the protocol are also provided in the supplementary file 1. Process control suspensions were handled together but were not irradiated. The irradiation was performed at the International Atomic Energy Agency laboratories, Seibersdorf, Austria.

2.3. Preparation of formalin-killed vaccine

Formalin-killed cells were prepared with *E. coli* PA14/17480/5-ovary as previously explained [26]. Cells treated with paraformaldehyde were mixed with Freund's adjuvant (1:1 ratio) before vaccination (Supplementary file 1).

2.3.1. Alamar blue assay

AlamarBlue™ cell viability assay (Invitrogen, Austria) was performed to determine the metabolic activity in irradiated, formalin-killed and process control (live cells) according to the manufacturer's instruction and the protocol described earlier [26]; details are provided in the supplementary file 1. Long term monitoring of metabolic activity was done in samples stored at 4 °C.

2.3.2. Electron microscopy

The transmission electron microscopy (TEM) was performed at the Vienna BioCenter Core Facilities, Vienna, Austria. *E. coli* PA14/17480/5-ovary cells were adsorbed onto the carbon side of a 400mesh Cu/Pd grid (Agar Scientific, UK) that were previously coated with a self-made 4 nm continuous carbon support film and glow-discharged in a BAL-TEC SCD005 sputter coater (BAL-TEC, Liechtenstein). After 1 min adsorption, unbound bacteria were washed off and grids were contrasted for 1 min with 0.25 % phosphotungstic acid (pH7). Random positions on the grids were inspected in a FEI Morgagni 268D transmission electron microscope (Eindhoven, the Netherlands) operated at 80 kV. Digital images were acquired using an 11-megapixel Morada CCD camera from Olympus-SIS (Germany).

2.4. MALDI-TOF MS

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was done from freshly prepared irradiated, formalin-killed and process control PA14/17480/5-ovary using Ultraflex-II MALDI-TOF mass spectrometer (Bruker Daltonik GmBH, Leipzig, Germany) as described [31].

2.5. Preparation of bacterial inoculum

The *lux*-tagged *E. coli* PA14/17480/5-ovary inoculum in trial 1 was prepared as explained earlier [32]. For other infection/challenge, bacteria were cultivated and washed as done for the preparation of irradiation, but the cell pellets were finally resuspended only in phosphate buffer saline (PBS; for detail refer to the supplementary file 1). Bacterial concentrations in inoculum before and after infection/challenge were quantified by colony forming unit (CFU) counts.

2.6. Animal experiments

The animal trials were approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012–TVG 2012 (license Number GZ 68.205/0195-V/3b/2019 with amendment GZ 2020–0.380.498; BMBWF GZ: 2021–0.276.453).

2.7. Animal trial 1

The first trial was conducted to investigate the homologous protection of birds following immunization with irradiated, live or formalin-killed *E. coli* PA14/17480/5-ovary. Freshly hatched 96 specified pathogen-free (SPF) layers (VALO Biomedica GmbH, Sachsenring, Germany) were equally divided into 6 groups (Fig. 1) and placed in separate isolators with negative pressure. At 7 days of age, birds in group 1 were immunized with irradiated *E. coli* suspension (1 ml/bird, concentration before irradiation: $7.8x10^8$ CFU/ml) whereas birds in groups 2 and 3 were infected with $7.9x10^8$ CFU/ml of *E. coli* (1 ml/bird) via aerosol route. Aerosolization was performed with a nebulizer as previously described [32].

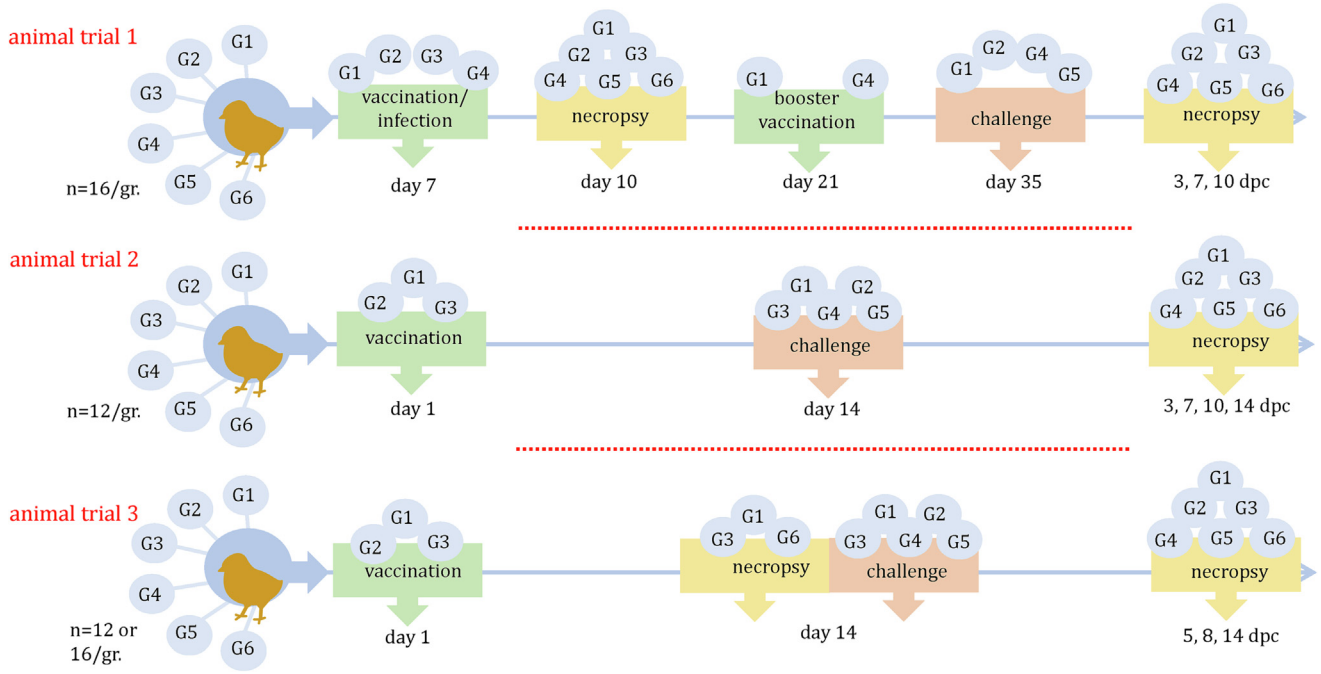

Fig. 1. Animal trials outline: "G" denotes for "Group". Animal trial 1: group 1: vaccinated with irradiated PA14/17480/5-ovary (first vaccination via aerosol and booster via oculonasal) and challenged. Group 2: infected with PA14/17480/5-ovary (aerosol) and challenged. Group 3: infected with PA14/17480/5-ovary (aerosol). Group 4: vaccinated with formalin-killed PA14/17480/5-ovary (intramuscular) and challenged. Group 5: challenge control. Group 6: negative control; *lux*-tagged *E. coli* PA14/17480/5-ovary was used for challenge. Four birds from each group except 3 birds from group 2 at 3 days post challenge were killed for sampling. Animal trial 2: group 1: vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA14/17480/5-ovary. Group 2: vaccinated with irradiated PA14/17480/5-ovary (oculonasal) and challenged with PA14/17480/5-ovary. Group 3: vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA16/13200-animal2/clone3. Group 4: homologous challenge control. Group 5: heterologous challenge control. Group 6: negative control. Three birds from each group except 2 birds from group 1 were killed for sampling after challenge. Animal trial 3: group 1: vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA14/17480/5-ovary. Group 2: vaccinated with irradiated PA14/17480/5-ovary and challenged with PA15/19103–3. Group 3: vaccinated with irradiated PA15/19103–3 (aerosol) and challenged with PA15/19103–3. Group 4: homologous challenge control. Group 5: heterologous challenge control. Group 6: negative control. Four birds were killed for necropsy and sampling.

The exposure setting, procedures, and particle dimensions are also explained in Supplementary Fig. 1. In group 4, each bird was injected intramuscular with 0.5 ml of formalin-killed bacterin (concentration before formalin treatment: $4.2x10^8$ CFU/ml). At ten days of age, 4 birds from each group were euthanized for necropsy and sampling. At three weeks of age, birds in groups 1 and 4 were boosted with irradiated cells via oculonasal route (0.5 ml/bird, concentration before irradiation: $3.1x10^8$ CFU/ml) and with formalin-killed *E. coli* (0.5 ml/bird, concentration before formalin treatment: $4x10^8$ CFU/ml), respectively. At five weeks of age, birds in groups 1,2,4 and 5 were challenged with $2x10^8$ CFU/ml of *lux*-tagged *E. coli* PA14/17480–5-ovary (1 ml/bird), via the aerosol route whereas birds in groups 3 and 6 were inoculated with the same volume of PBS using the same route. At 3,7- and 10-days post challenge (dpc), 4 birds from each group, except group 2 in which one bird died before challenge, were euthanized for samplings. Clinical signs were recorded daily and blood samples were collected weekly from all birds.

2.8. Animal trial 2

In the second trial, the efficacy of a single application of the prototype vaccine candidate via different routes and protection against homologous or heterologous challenges were evaluated. For this, day-old SPF layers (VALO Biomedica GmbH) were divided into 6 groups with 12 birds each (Fig. 1). At day one of life, birds in groups 1 and 3 were inoculated with irradiated PA14/17480–5-ovary by aerosolization (concentration before irradiation: $3.8x10^8$ CFU/ml) and birds in group 2 were administered with the same suspension via the oculonasal route. At two weeks of age, challenge was performed with $4.2x10^8$ CFU/ml of PA14/17480–5-ovary (groups 1,2,4) or with $3.2x10^8$ CFU/ml of PA16/13200-animal2/clone3 (groups 3,5). Negative control birds in group 6 were inoculated with PBS. The volumes used for vaccination and challenge were the same as described in trial 1. Following challenge, 3 birds/-group, except group 1 with one dead bird prior challenge, were killed at 3,7,10 and 14 dpc for necropsy and sampling. Clinical signs were observed daily. Blood samples were collected from surplus birds killed immediately after hatch, from all birds at the day of challenge and from killed birds before doing necropsy.

2.9. Animal trial 3

The third trial was conducted to evaluate the efficacy of irradiated *E. coli* against a heterologous challenge with O78:K80 strain and to investigate the efficacy of two irradiated strains against respective homologous challenges. In total, 84 SPF layers (VALO Biomedica GmbH) were allocated into 6 groups (Fig. 1). At first day of life, birds in groups 1 and 2 were vaccinated with irradiated PA14/17480–5-ovary (concentration before irradiation: $3.7x10^8$ CFU/ml) while birds in groups 3 were immunized with irradiated PA15/19103–3 (concentration before irradiation: $5.4x10^8$ CFU/ml) via aerosol route. At 14 days of age, 4 birds each from groups 1,3 and 6 were killed for sampling. Afterwards, remaining birds were challenged with $3.6x10^8$ CFU/ml of PA14/17480–5-ovary (groups 1,4) or $7.7x10^8$ CFU/ml of PA15/19103–3 (groups 2,3,5). Negative control birds (group 6) were inoculated with PBS. The vaccination and challenge procedures were followed as in previous trials. After challenge, 4 birds were killed at 5,8 and 14 dpc for sampling. Clinical signs were recorded daily and blood samples were collected from surplus birds after hatch and from chickens killed after challenge.

2.10. Histopathology

Samples from lungs, airsac, spleen, liver and heart were collected during necropsy and fixed in 10 % neutral buffered formalin. Tissue samples were embedded in paraffin, sectioned into 5 µm slices, and stained with Hematoxylin-Eosin stain. Lesions were categorized from lesion score (LS) 0 to 2 (Supplementary Table 1) applying a recently established scoring scheme [32].

2.11. Bacteriology

Aseptically collected blood samples were enriched in Lenox L Broth Base (Invitrogen, Vienna, Austria) overnight at 37 °C and direct plating was done on agar plates. Lungs, liver and spleen were processed for CFU count while heart and airsac were directly streaked on agar plates for bacterial re-isolation. Absolute bacterial quantification was determined by CFU count in duplicate plates. Following overnight incubation of plates at 37 °C, growth of *E. coli* was recorded (direct plating) or colonies were counted (CFU count). MacConkey agar plates were used for the growth of *E. coli* except in the animal trial 1 where the challenged strain *lux*-tagged *E. coli* PA14/17480–5-ovary was selectively grown on LB agar plates with erythromycin [29].

2.12. ELISA

Serum antibody titers against *E. coli* challenge strains were measured with separate in-house customized enzyme linked immunosorbent assays (ELISAs) as described before [33]. Checkerboard titration was done to select the best combination of the bacterial concentration for coating and serum dilution. Separate *E. coli* strains with the concentration of 8 log CFU/ml were used for coating 96-well plates depending on the type of serum tested. On the next day, plates were dried and each well was treated with blocking buffer for one hour. After removing the blocking buffer, test sera diluted at 1:200 were conjugated with a secondary antibody (Goat-anti-Chicken IgG-HRP) at dilution 1:5000. Color reaction was initiated by adding tetramethylbenzidine substrate and stopped with 0.5 M H_2SO_4. Optical densities (ODs) were measured with an ELISA reader at 450 nm.

2.13. Statistical analysis

For statistical analysis, all the observations after challenge were grouped together. The patterns of *E. coli* positive/negative organs in CFU and direct plating were treated as a binary response (negative = 0, positive = 1) and analyzed with binary logistic regression models [34]. Microscopic LSs treated as an ordered categorical response were analyzed with ordinal logistic regression models (package *ordinal*, function *clm*) [35] after verification of the proportional odds assumption via a likelihood ratio test using the function *nominal_test* (package *ordinal*). In case only two microscopic LSs were observed, they were treated as a binary response in logistic regression models. Overall significance of each fixed categorical effect at an alpha cut off of 5 % was evaluated via a likelihood ratio test using the function *anova* in R, comparing the full model to a reduced model without that fixed effect. When two fixed categorical effects were present (e.g. vaccination and challenge), their interaction was evaluated via a similar likelihood ratio test and multicolinearity was assessed with variance inflation factors (package *car*, function *vif*) [36]. Pairwise comparisons between the levels of a fixed categorical effect in a model were conducted via the estimated marginal means (emmeans, package *emmeans*, function *emmeans*) [37]. We declared significance at an alpha cut off of 5 % after multiple testing corrections (R function *p.adjust* with the Bonferroni-Holm correction).

For interpretation, emmeans in logistic regression models are log-odds values (odds of a higher response level). When the log-odds values are positive, the predicted probability for a higher response level (e.g. organs positive in bacteriology or high lesion score) is larger than the predicted probability for a lower response level. Interpretation should be made exactly in the opposite direction when the log-odds values are negative. When two emmean values are compared, the greater emmean value corresponds to a greater predicted probability for a higher response level.

The average OD values in Alamar Blue assay and ELISA among groups were compared using the *t*-test and one-way ANOVA with Tukey's post hoc test, respectively (IBM® SPSS® version 25; IBM cooperation, New York, USA).

3. Results

3.1. In vitro characteristics of irradiated cells

Irradiation was performed with 1.2 Kgy of Cobalt-60, which completely inhibited bacterial multiplication even after enrichment.

Almost no metabolic activity remained in formalin-killed cells (Fig. 2A). In contrary, percentage reduction of Alamar Blue in irradiated and process control PA14/17480/5-ovary were similar for at least two weeks (Fig. 2A, B, C). The percentage reduction values were much lower in non-treated PA15/19103, which slightly increased after irradiation but did not reach the level of PA14/17480/5-ovary (Fig. 2D).

The TEM showed that the outer cellular structure (bacterial cell integrity and fimbria) of PA14/17480/5-ovary process control was similar to irradiated cells (Fig. 2E, F).

The protein profile spectra including all major peaks of process control and irradiated PA14/17480/5-ovary were similar (Fig. 2G). Consequently, both types of cells produced same log score values for reliable species identification with MALDI-TOF MS. No such peaks were observed in formalin-killed cells.

3.2. In vivo experiments

3.2.1. Clinical signs

In trial 1, mild drowsiness and ruffled feathers were observed in 2–5 birds/day in infected groups 2 and 3 starting from the first day until 16 days post infection (dpi). Such clinical signs were inconsistent and not always the same birds were affected. One bird from group 2 was euthanized 13 dpi due to severe symptoms such as, difficulty in breathing, dropped wings and inability to move.

In trial 2, one bird of group 1 died 5 days post vaccination (dpv). After challenge, 2–4 birds/day in groups 2,4 and 5 showed inconsistent mild clinical signs as in trial 1. At 8 dpc, intense clinical signs appeared in one bird from group 4 which was killed 10 dpc.

In trial 3, all birds from groups 1 and 6 were healthy. In group 2, one bird showed weak clinical signs. In groups 3,4 and 5, mild to moderate clinical signs were noticed in 3,5 and 4 birds, respectively.

3.2.2. Macroscopic lesions

In trial 1, at 3 dpi, mild to severe airsacculitis and perihepatitis were seen in birds from groups 2 and 3 (Supplementary Table 2, Fig. 3A). The euthanized bird from group 2 at 13 dpi had severe perihepatitis, pericarditis and airsacculitis. At 3 dpc, mild pericarditis was seen in 1 bird in group 2 whereas fibrinous pericarditis (n = 1) and cloudy airsac (n = 2) were recorded in birds of group 5 at 7 dpc. No lesions were observed in birds from groups 1 and 4 that were vaccinated with irradiated and formalin-killed *E. coli* respectively.

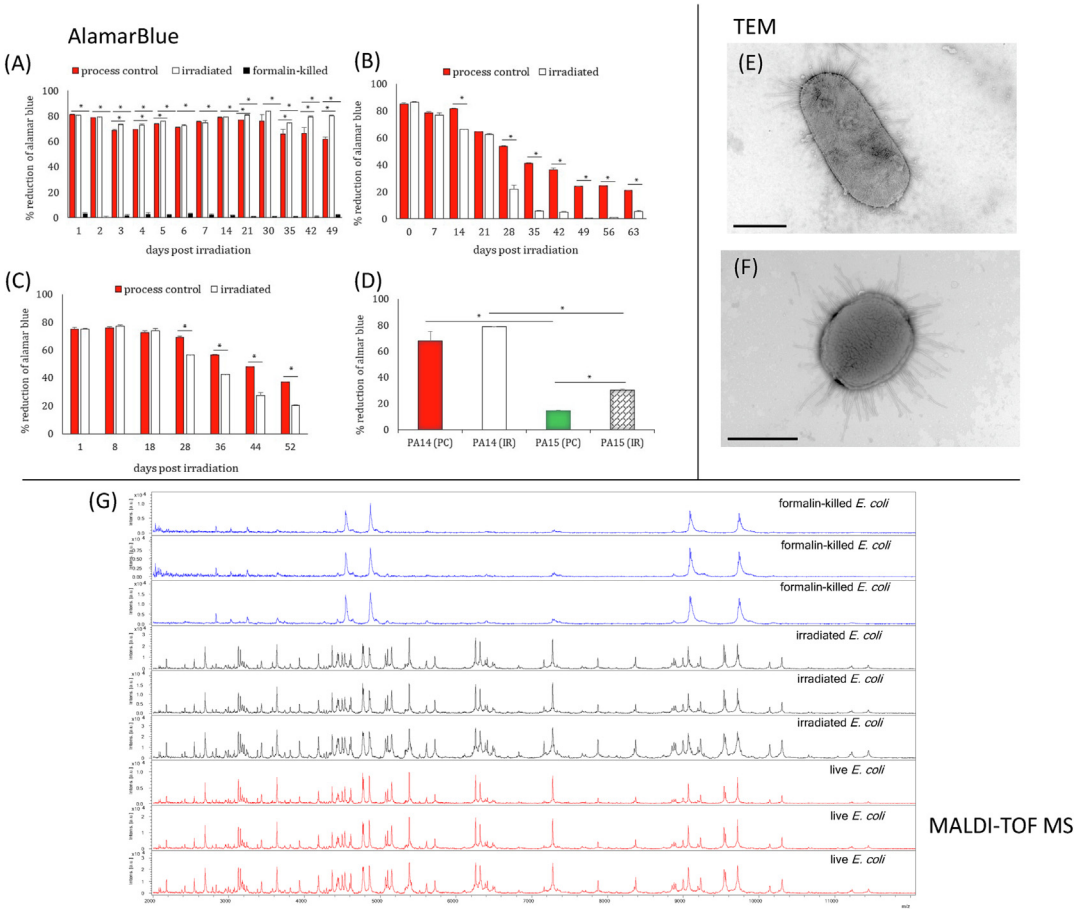

Fig. 2. *In vitro* properties of irradiated *E. coli*. A, B, C: percentage reduction of Alamar Blue in process control and treated PA14/17480/5-ovary in three separate experiments. D: the same measurement in process control (PC) and irradiated (IR) PA14/17480/5-ovary (PA14) and PA 15/19103–3 (PA15) at 1-day post irradiation; asterisks denote for statistically significant differences on the day of measurement, $p \leq 0.05$. E: transmission electron microscopy of process control *E. coli* PA14/17480/5-ovary, and F: the same strain irradiated by gamma radiation; scale bar 1 µm. G: Protein profiling with matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI TOF-MS), three samples of live *E. coli* PA14/17480/5-ovary (process control) and the same strain after irradiation or formalin treatment were analyzed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

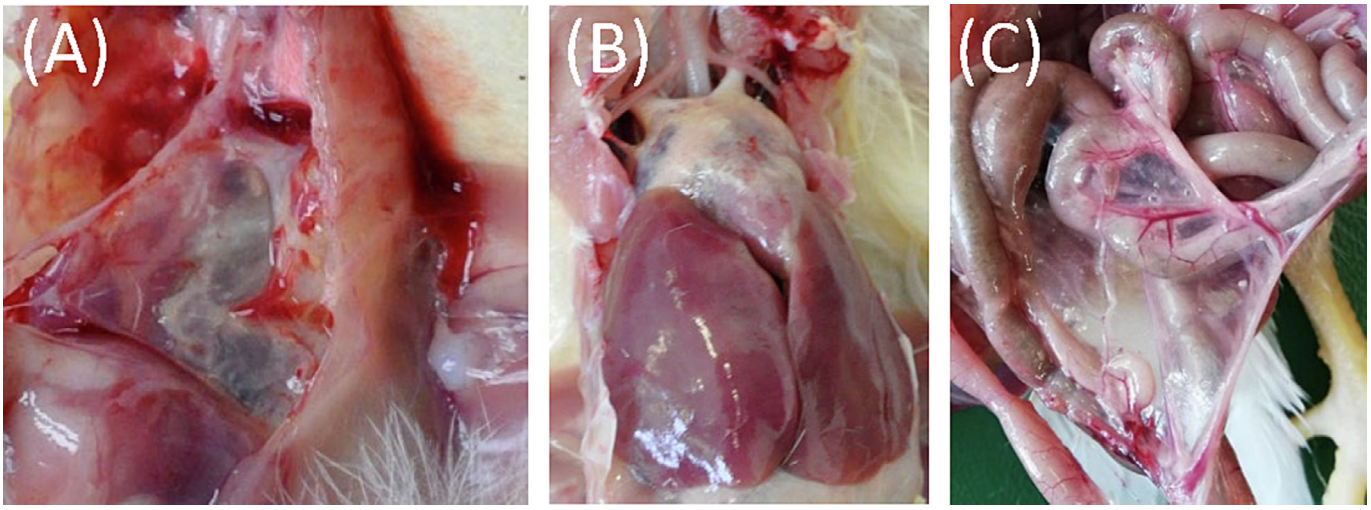

Fig. 3. Macroscopic lesions. A: airsacculitis; trial 1, group 2, infected with PA14/17480/5-ovary, at 3 days post infection. B: perihepatitis and pericarditis; trial 2, group 4, challenge control, at 10 days post challenge (dpc). C: airsacculitis (cloudy air sac) and slight peritonitis; trial 2, group 4 at 10 dpc.

In trial 2, unabsorbed yolk was seen in the bird which died 5 dpv. Birds in group 1 did not show lesions except one with moderate airsacculitis at 10 dpc (Supplementary Table 2). In group 2, mild to moderate airsacculitis (n = 3), perihepatitis (n = 2) and pericarditis (n = 1) were observed. No lesions were seen in birds of group 3. In the homologous challenge control group (group 4),

lesions such as airsacculitis (n = 5), perihepatitis (n = 1), pericarditis (n = 2) and peritonitis (n = 1) were observed (Fig. 3B, C). Likewise, airsacculitis (n = 3), perihepatitis (n = 3) and pericarditis (n = 2) were also observed in the heterologous challenge control group (group 5) but lesions were milder than in group 4.

In trial 3, no lesions were observed in birds of groups 1 and 6 (Supplementary Table 2). In group 2, two birds showed moderate airsacculitis with or without mild perihepatitis 5 dpc and such lesions were recorded in a single bird 8 dpc. Birds in group 3 showed mild to moderate airsacculitis (n = 5), perihepatitis (n = 2) or pericarditis (n = 4). Birds in groups 4 and 5 had the most severe lesions until the end of the trial. In group 4, peritonitis was additionally observed in 3 birds (2 at 5 dpc, 1 at 8 dpc).

3.2.3. Histopathological lesions

In trial 1, at 3 dpi, microscopic lesions were mostly seen in infected birds (Supplementary Table 3). Prior to challenge, all birds except one in group 1 with LS 1 in airsac and spleen were devoid of pathological lesions. After challenge, the number of birds with LS 1 or 2 in systemic organs was the highest in group 5, which is the challenge control. The emmean values in all organs were the lowest in group 1, and the difference observed in airsac between groups 1 and 2 that were vaccinated with irradiated and live *E. coli*, respectively, was statistically significant (Fig. 4).

In trial 2, most birds with lesions were in group 4 (Supplementary Table 4). Among groups 1 (aerosol-vaccinated and challenged), 2 (oculonasal-vaccinated and challenged) and 4 (homologous challenge control), emmean values in lungs and airsac were the lowest in group 1 whereas in spleen and heart the lowest values were observed in group 2 (Fig. 5A). Comparison of group 1 with 4, and group 3 (vaccinated and heterologous challenged) with 5 (heterologous challenge control) showed that lesions in lungs, heart and airsac were reduced in vaccinated birds after challenge with both homologous and heterologous strains, and the differences were statistically significant in airsac (Fig. 5B).

In trial 3, birds killed before challenge in groups 1,3 and 6 did not show any lesions. After challenge, microscopic lesions were mostly observed in challenge controls whereas vaccinated and challenged groups showed varied results (Supplementary Table 5). The emmean values were compared between groups 1 and 4 as well as 2 and 5 (Fig. 6A). Birds in groups 1 and 2 were vaccinated with irradiated PA14/17480/5-ovary, and challenged either with PA14/17480/5-ovary or PA15/19103–3. Groups 4 and 5 were challenge controls for PA14/17480/5-ovary and PA15/19103–3, respectively. The emmean values, most prominently in lungs and airsac were lower in vaccinated birds. The values were statistically significant in lungs and airsac in case of homologous challenge. A similar comparison was made among groups 2,3 and 5that were inoculated with irradiated PA14/17480/5-ovary, PA15/19103–3 or PBS only but all were challenged with PA15/19103–3 (Fig. 6B). The occurrence of histopathological lesions in group 2 was the least in lungs, spleen, heart and airsac followed by groups 3 and 5.

3.2.4. Re-isolation of E. coli

In trial 1, at 3 dpi, majority of infected birds in groups 2 and 3 were positive for *E. coli* (Supplementary Table 6). In group 1, only one positive case was observed prior challenge in lungs. Pure cultures of *E. coli* were isolated from lungs, airsac, heart, and liver of the euthanized bird 13 dpi. After challenge, the re-isolation pattern was comparable in all vaccinated groups. Particularly, in group 1 immunized with irradiated *E. coli*, 3,2 and 1 birds were positive for *lux*-tagged PA14/17480/5-ovary in lungs, airsac and blood, respectively, while all other systemic organs were negative. In contrast, 5,1,1,3,4 and 5 birds were positive in lungs, airsac, heart, liver, spleen and blood of birds in group 5. Thus, the frequency of *E. coli* positive lungs and the systemic spread was higher in challenge controls than in vaccinated birds.

In trial 2, *Pseudomonas aeruginosa* was cultured from yolk sac of dead bird. After challenge, lungs were mostly positive for *E. coli* in all challenged birds, irrespective of their vaccination status (Supplementary Table 7). All except one bird vaccinated via aerosol route were negative in systemic organs. Comparing groups vaccinated via different routes with the homologous challenge control, the likelihood of bacterial re-isolation was the highest in group 4 (Fig. 7A). The aerosol vaccinated group had lower emmeans as compared to the oculonasal vaccinated group. Statistically significant difference was observed between groups 1 and 4 in the liver. Furthermore, comparison between groups 1 and 3 with 4 and 5, respectively, showed that the likelihood of positive re-isolation from lungs, liver, heart, airsac and blood was less in vaccinated groups than in challenge controls (Fig. 7B).

In trial 3, irrespective of vaccination status, lungs of majority of challenged birds were positive, similar to trials 1 and 2 (Supplementary Table 8). However, systemic bacterial spread was very limited in groups 1 and 2 (both vaccinated with PA14/17480/5-ovary), which also showed the lowest emmean values as compared to respective challenge controls, groups 4 (homologous) and 5 (heterologous) (Supplementary Table 8, Fig. 8A). The differences between groups 1 and 4 in lungs and spleen were statistically significant whereas comparison of groups 2 and 5 showed such significance in liver, spleen, heart and airsac. In group 3, colonization pattern was similar to group 5 (Supplementary Table 8) and emmean values were higher in lungs, airsac, liver and spleen than in group 1 (Fig. 8B). Compared to group 5, differences in liver, spleen, heart and airsac were significant in group 2 and in heart in group 3.

3.2.5. Antibody titers

In trial 1, antibody titers were not influenced due to irradiated *E. coli* in birds of group 1 (Fig. 9A). Birds vaccinated with formalin-killed bacteria (group 4) and infected at day 7 (groups 2 and 3) showed the maximum antibody level before challenge, which was not provoked afterwards. In group 1, antibody titer increased after 7 dpc and reached to the maximum at 10 dpc. At the final time point, similar antibody responses were observed in birds of groups 1 to 5.

In trial 2, sera of birds challenged with PA16/13200-animal2/clone 3 were tested in plates coated with PA14/17480/5-ovary but no cross reactivity was seen (data not shown), thus, homologous strains were considered for coating. Sera from negative control birds were tested in separate plates coated with PA14/17480/5-ovary (homol.) or PA16/13200-animal2/clone 3 (heterol.). Birds in group 6 remained with low antibody titers independent of the coating antigen (Fig. 9B). One-week post challenge only birds in challenge control group 5 showed a significantly higher antibody level in comparison to control birds, which did not rise considerably until the end of the study. At 10 dpc antibody levels were already high in all challenged groups, except group 3. Finally, at 14 dpc, the values in all challenged groups were significantly higher than in the negative control.

In trial 3, antibody titers increased following challenge of birds in consistent with observations in previous two trials (Supplementary Fig. 2). Again, challenge control birds (groups 4 and 5) showed a tendency of immediate increase of antibodies already 5 dpc. At later two time points, all challenged groups exhibited similar titers.

4. Discussion

Colibacillosis is a well-recognized disease in poultry with significant impacts on animal welfare, production and public health but

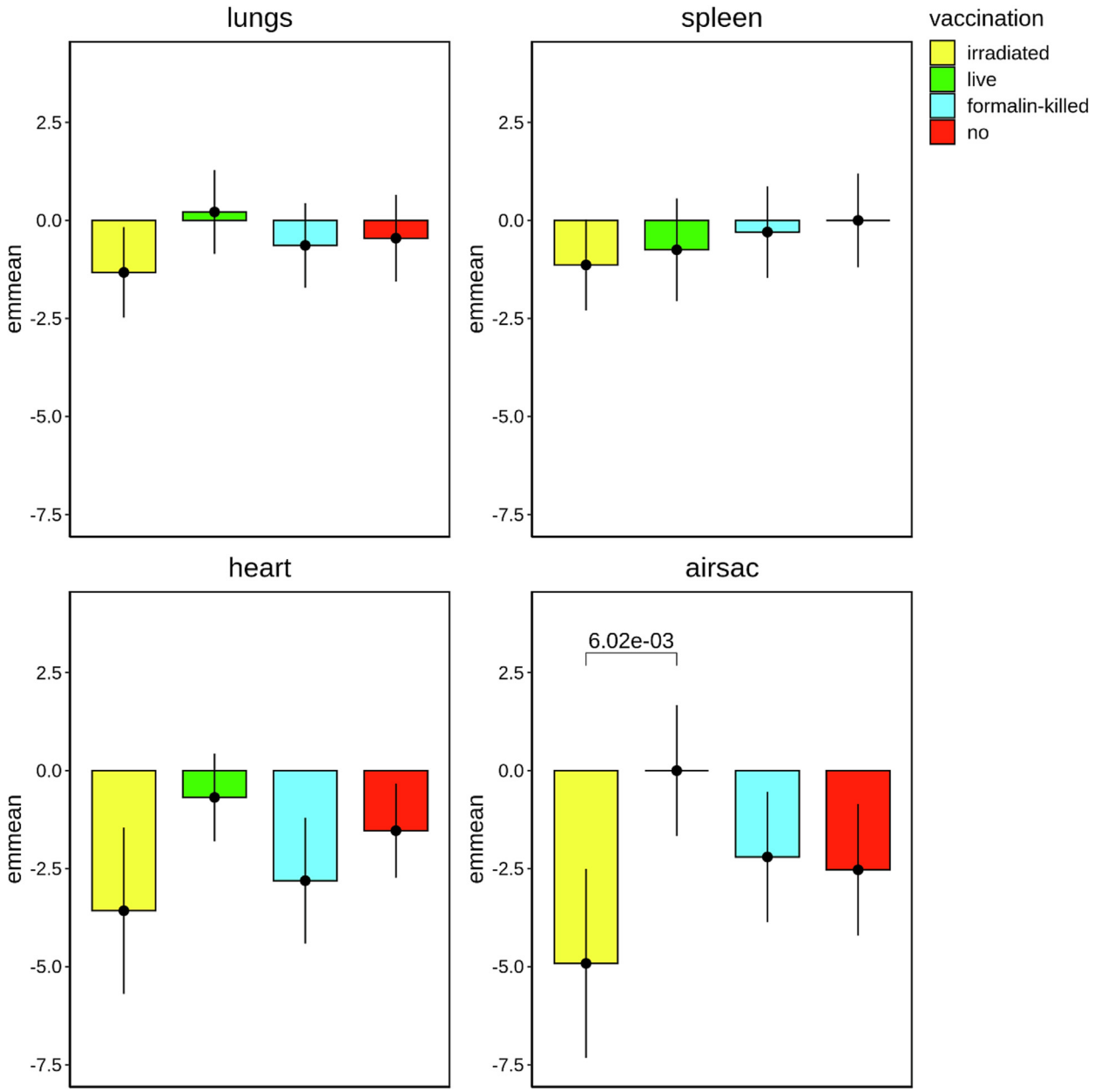

Fig. 4. Histology, trial 1: Emmean values (log-odds values) from the ordinal logistic regression models used to evaluate differences in histopathology lesion scores after challenge. Group 1 (yellow): vaccinated with irradiated PA14/17480/5-ovary (first vaccination via aerosol and booster via oculonasal) and challenged. Group 2 (green): infected with PA14/17480/5-ovary (aerosol) and challenged. Group 4 (light blue): vaccinated with formalin-killed PA14/17480/5-ovary (intramuscular) and challenged. Group 5 (red): challenge control. Challenge was done with *lux*-tagged *E. coli* PA14/17480/5-ovary. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the disease mitigation by vaccination has always been a challenge. In the present study, we conducted a series of *in vitro* and *in vivo* experiments in order to investigate the efficacy of irradiated *E. coli* cells to prevent the disease and the suitability for aerosol application in young chickens. Irradiation has long been used for sterilization of food and food products [22]. Irradiated bacteria do not multiply but retain metabolic and cellular functions such as production of major metabolites without affecting the cell wall-associated peptidoglycan pathway [38], undamaged outer membrane structure [39], ability to allow bacteriophages propagation [40] and possession of *de novo* transcriptional and gene expression activity [24,41]. Consequently, several previous studies showed that irradiated bacteria, eg. *Streptococcus pneumoniae*, *Listeria monocytogenes* or *Brucella melitensis* elicited better immune response in animals as compared to heat or formalin-killed bacterins [23,25,42]. In most cases, MALDI-TOF MS can correctly identify irradiated bacteria although biotyper scores might be lowered [43]. Consistent with previous literatures, outer cellular structure and protein spectra in irradiated *E. coli* in the present study were similar to non-treated cells. In MALDI-TOF MS, very few peaks observed in formalin-killed cells correlate with the fact that formalin causes permanent covalent bond with cellular proteins limiting their ionization [44]. Likewise, irradiation preserved the bacterial metabolic activity, which is a well understood fact [21]. Gradual reductions of metabolic activities after irradiation in experiments 2 and 3 are similar to previous reports [39,40]. Discrepancies seen in experiment 1, in both irradiated and process control, could be due to the differences in rate of cell death during storage. Interest-

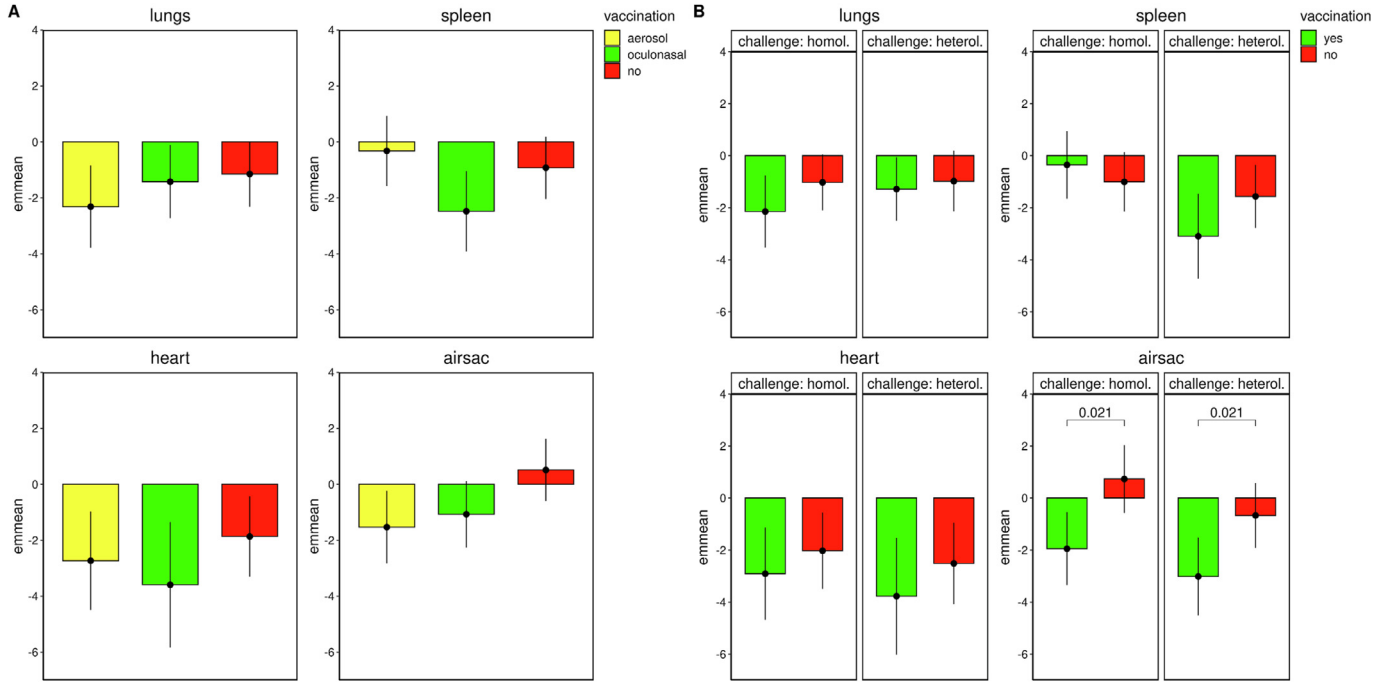

Fig. 5. Histology, trial 2. A: Emmean values (log-odds values) from the ordinal logistic regression models used to evaluate differences in histopathology lesion scores after challenge. Group 1 (yellow): vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA14/17480/5-ovary. Group 2 (green): vaccinated with irradiated PA14/17480/5-ovary (oculonasal) and challenged with PA14/17480/5-ovary. Group 4 (red): challenge control. B: Group 1 was compared with group 4, and group 3, vaccinated with irradiated PA14/17480/5-ovary and challenged with PA16/13200-animal2/clone3 was compared with group 5, which was not vaccinated but challenged with PA16/13200-animal2/clone3. Vaccinated and challenged groups are shown in green whereas challenge controls are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ingly, the rate of alamarBlue reduction in two *E. coli* strains differed, which corelated with their efficacy as discussed below.

In contrast to live *E. coli*, absence of lesions in birds immunized with irradiated *E. coli* in trial 1 ensured its safety. After challenge,

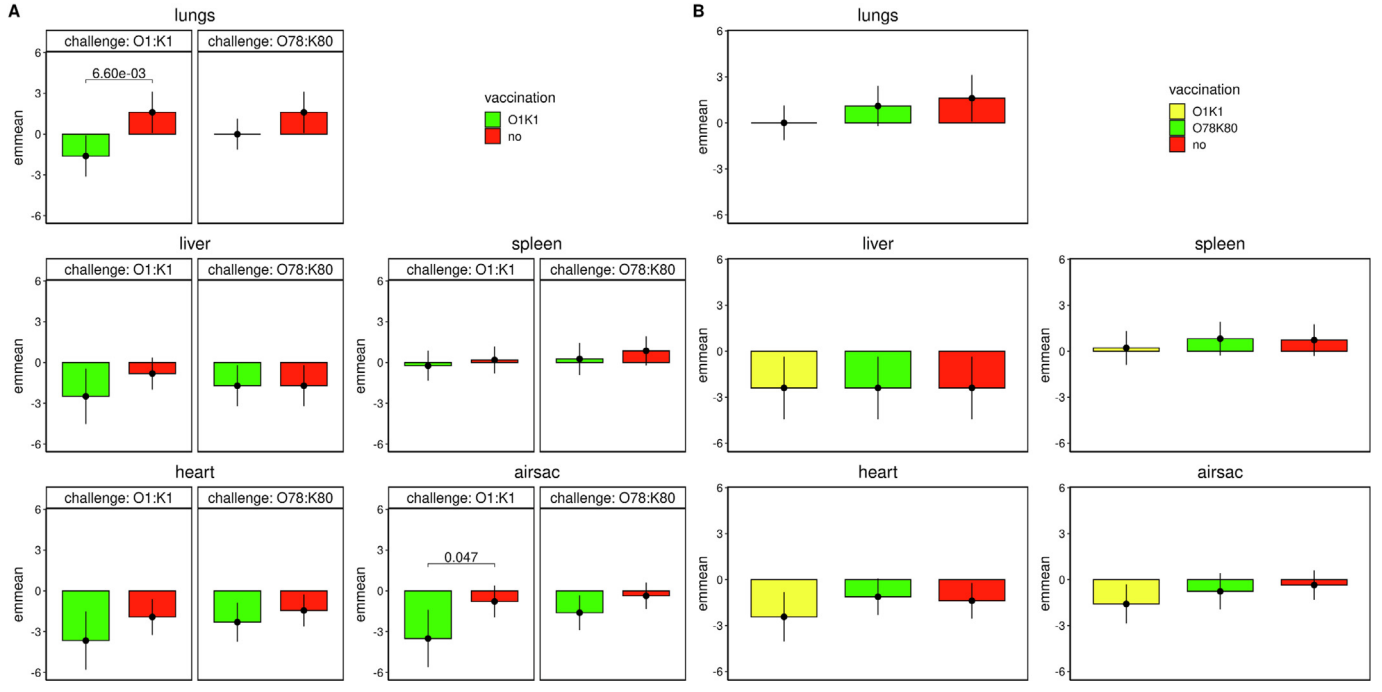

Fig. 6. Histology, trial 3. A: Emmean values (log-odds values) from the ordinal logistic regression models used to evaluate differences in histopathology lesion scores after challenge. Group 1: vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA14/17480/5-ovary. Group 2: vaccinated with irradiated PA14/17480/5-ovary and challenged with PA15/19103–3. Group 4: challenged with PA14/17480/5-ovary. Group 5: challenged with PA15/19103–3. Vaccinated groups (shown in green) were compared with their respective challenge controls (in red). B. Comparison among group 2 (yellow), group 3 (green), which was vaccinated with irradiated PA15/19103–3 (aerosol) and challenged with PA15/19103–3, and group 5 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

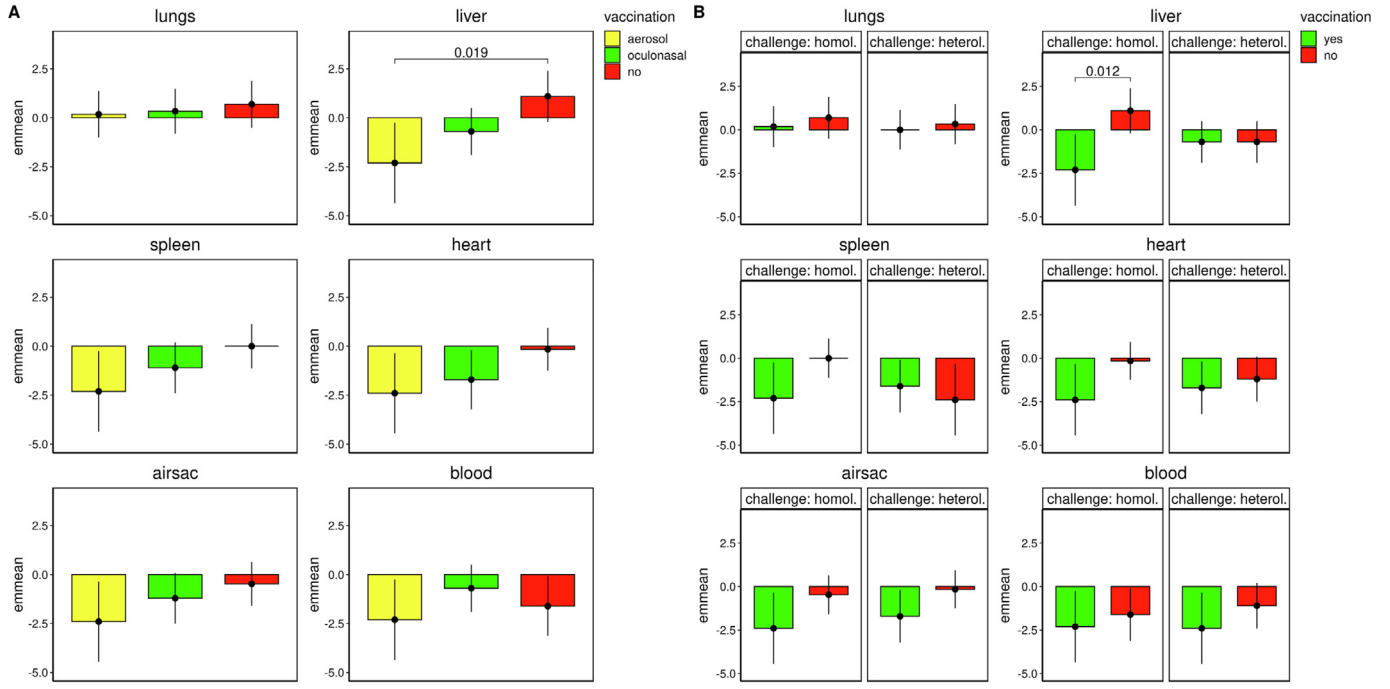

Fig. 7. Bacteriology, trial 2. A: Emmean values (log-odds values) from the binary logistic regression models used to evaluate differences in *E. coli* re-isolation after challenge. Group 1 (yellow): vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA14/17480/5-ovary. Group 2 (green): vaccinated with irradiated PA14/17480/5-ovary (oculonasal) and challenged with PA14/17480/5-ovary. Group 4 (red): challenge control. B: Group 1 was compared with group 4, and group 3, vaccinated with irradiated PA14/17480/5-ovary and challenged with PA16/13200-animal2/clone3, was compared with group 5, which was not vaccinated but challenged with PA16/13200-animal2/clone3. Vaccinated and challenged groups are shown in green whereas challenge controls are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

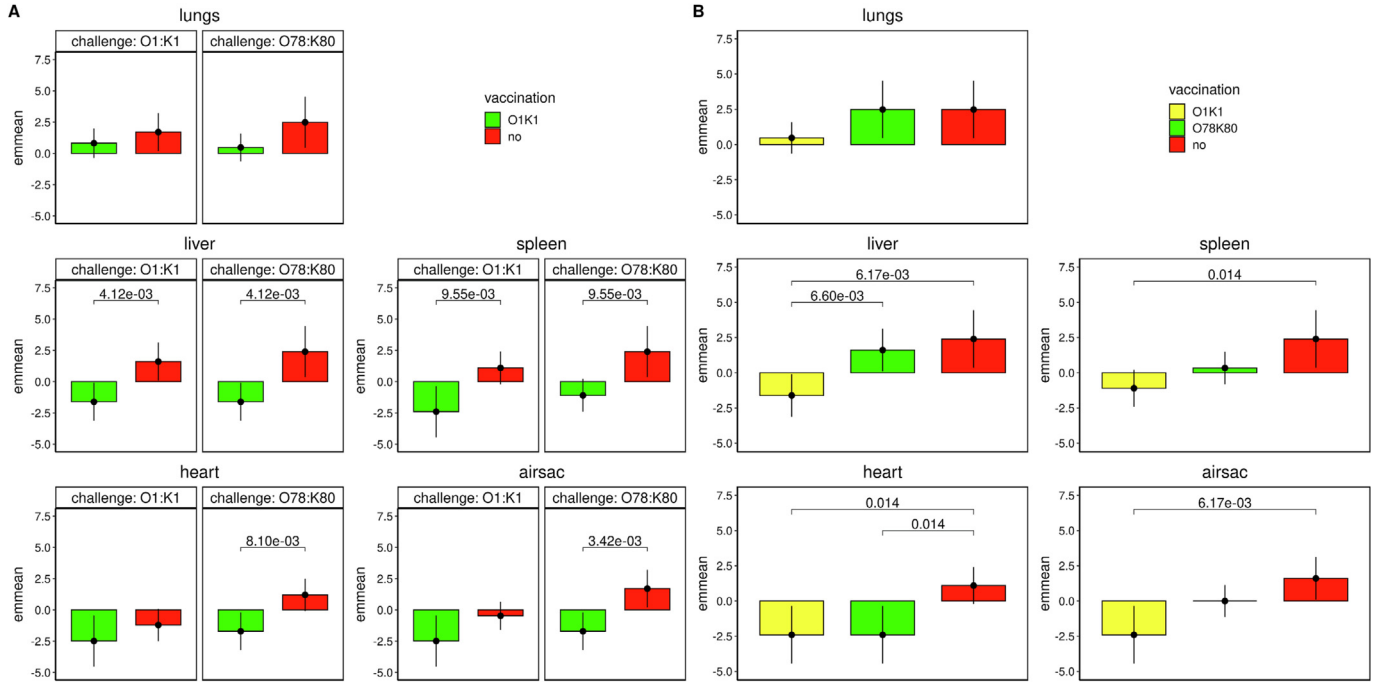

Fig. 8. Bacteriology, trial 3. A: Emmean values (log-odds values) from the logistic regression models used to evaluate differences in *E. coli* re-isolation after challenge. Group 1: vaccinated with irradiated PA14/17480/5-ovary (aerosol) and challenged with PA14/17480/5-ovary. Group 2: vaccinated with irradiated PA14/17480/5-ovary and challenged with PA15/19103–3. Group 4: challenged with PA14/17480/5-ovary. Group 5: challenged with PA15/19103–3. Vaccinated groups (shown in green) were compared with their respective challenge controls (in red). B. Comparison among group 2 (yellow), group 3 (green), which was vaccinated with irradiated PA15/19103–3 (aerosol) and challenged with PA15/19103–3, and group 5 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the lowest emmean values with regards to histopathology LSs in birds inoculated with irradiated *E. coli* indicated their protection, as efficient as live infection, which was also supported by the limited bacterial re-isolation from blood and systemic organs. Previ-

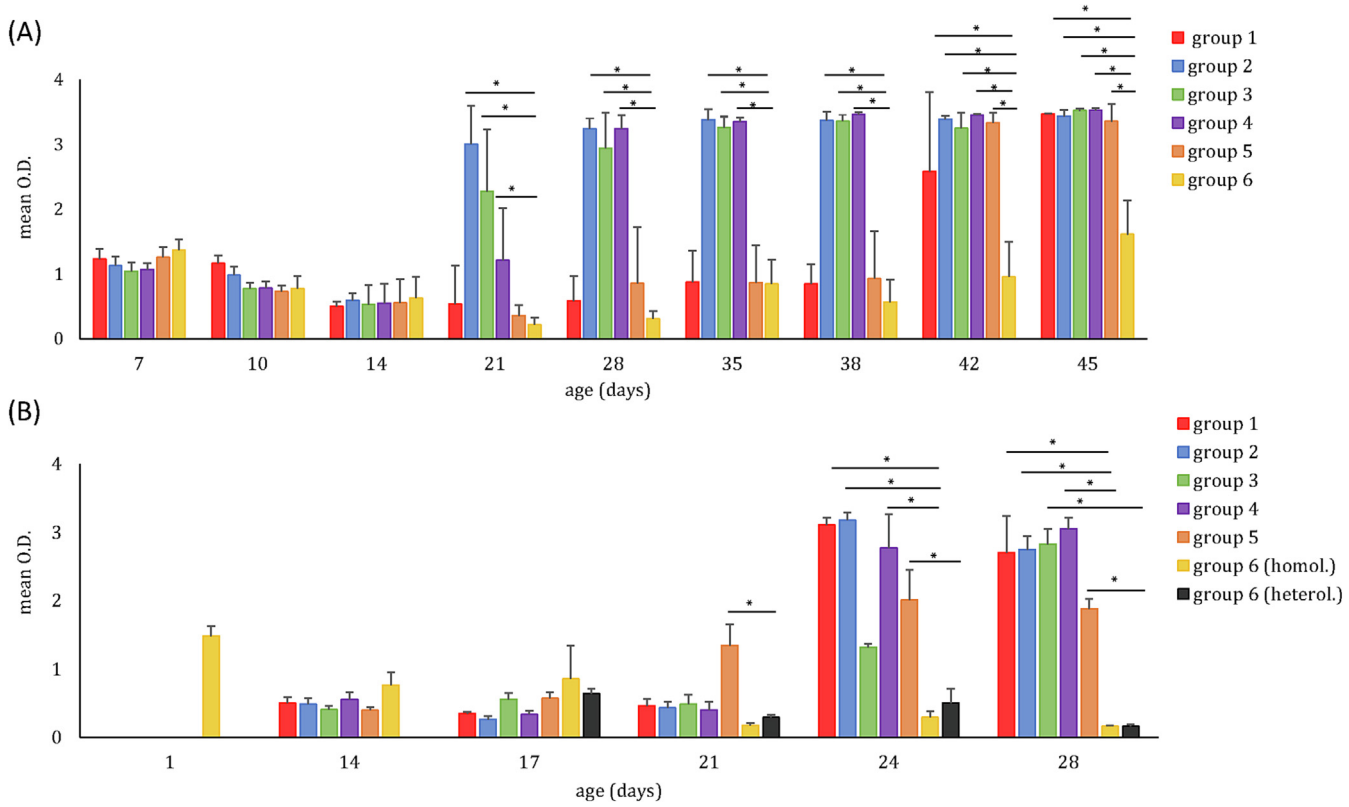

Fig. 9. Quantification of IgY antibodies. A: level of IgY antibodies measured against PA14/17480/5-ovary in animal trial 1; for trial design refer to Fig. 1; all plates were coated with PA14/17480/5-ovary. B: level of antibodies against PA14/17480/5-ovary or PA16/13200-animal2/clone 3 in animal trial 2. Plates used for testing sera from groups 1, 2 and 4 were coated with PA14/17480/5-ovary and those for groups 3 and 5 were coated with PA16/13200-animal2/clone; at 17, 21, 24 and 28 days, PA14/17480/5-ovary (homol.) or PA16/13200-animal2/clone (heterol.) were used for coating to test sera from negative control birds; for trial design refer to Fig. 1. Asterisks denote statistically significant differences. $p \leq 0.05$.

ously, it was shown that subcutaneous injections of irradiated or formalin/heat killed + irradiated *E. coli* were not effective, but the protection was significant with ultrasonicated bacterial cells followed by irradiation [45]. Thus, the method of preparation and application of irradiated bacteria might influence its efficacy in the host.

The second trial highlighted importance of the vaccine delivery route as birds vaccinated via aerosol showed better results in terms of severity of lesions and pattern of bacterial colonization in systemic organs than those immunized oculonasally. In the past, several routes such as oral, intramuscular, intratracheal or spray were used for the delivery of live or live attenuated *E. coli* vaccines [3]. In order to facilitate deep penetration of vaccine particles into the respiratory tract in day-old chicks, fine spray or aerosol methods were preferred [18,46]. In young chickens, *E. coli* can efficiently reach to lower respiratory organs such as lungs or airsac after aerosolization [32]. Previously it was shown that a live attenuated vaccine increased cellular immune response, mainly $CD4^{+}TCRV\beta1^{+}$ cells that are related to the respiratory mucous membrane [47]. For enhancement of such mucosal immunity, the vaccine particles should come in contact with the maximum surface area of the upper as well as lower respiratory organs [48]. Keeping it altogether, the difference observed in the present study with two vaccination routes should be attributed to differently administered vaccine particles into respiratory organs.

The second trial also indicated that irradiated PA14/17480/5-ovary was able to protect birds against challenge with a homologous and a heterologous strain isolated from a systemic organ of a diseased bird, commonly called as avian pathogenic *E. coli* (APEC). The protection was not very prominent because the heterologous challenge strain was not as pathogenic as PA14/17480/5-ovary. Thus, several strains isolated from bone marrow of broilers were tested for their pathogenicity in chickens in order to choose a more virulent field isolate (data not shown). Based on this, PA15/19103–3 was selected for vaccine preparation and heterologous challenge. Subsequently, the third trial reconfirmed the efficacy of PA14/17480/5-ovary to prevent lesions, prominently in lungs and airsac as well as bacterial colonization in all the organs tested after homologous or heterologous challenges signifying the broader efficacy of the selected *E. coli* strain. Interestingly, irradiated PA15/19103–3 was found to be less suitable for preventing systemic spread of bacteria and lesions, especially in the lower respiratory organs even after the homologous challenge. As mentioned above, these two strains also differed in terms of metabolic activity, which is essential for stimulation of host immune response. A clear correlation observed between the metabolic activity in *E. coli* stains and their protection ability in birds is worth of further study; however, the observation already emphasized the importance of strain selection.

The irradiated *E. coli* when administered via the aerosol route, despite being protective, did not provoke an antibody response. A previous study reported high antibody titer after subcutaneous administration of ultrasonicated + irradiated *E. coli* [45], and the observed differences could be attributed to routes of application. Usually, the protection with a live attenuated *E. coli* vaccine does not correlate with the circulating antibodies [47]. Furthermore, based on *ex vitro* stimulation assay of mononuclear cells, we recently found that the initial *T*-cell response could be the hallmark of protection during *E. coli* infection, and the expression of IFN-γ and IL-17A against live and irradiated APEC were very similar

[26]. Thus, humoral immunity in birds seems to be less important after immunization with an irradiated vaccine.

Gamma radiation used in the present study needs special safety measures. But, it is expected that the proof-of-concept demonstrated in a laboratory set up in the present study can be easily transferred to future studies using newer technologies involving X-ray [49] and ebeam [50], which also work in the same principle and are very safe for commercial operation. The composition can be further improved considering optimum concentration, suitable adjuvants or formulation such as dry powder.

In conclusion, we have demonstrated that a single application of non-adjuvanted irradiated *E. coli* in chickens, delivered via aerosol route is broadly protective against homo-or heterologous challenges with isolates obtained from layers and broilers. The findings should be a valuable addition in the portfolio of *E. coli* vaccine for controlling colibacillosis, having several advantages with regards to safety, production and feasibility for mass application.

Funding

SP received the Bright Spark grant from the University of Veterinary Medicine Vienna, Austria, which partially supported the study.

Data availability

The data supporting the findings of this study are available within the manuscript and supplementary materials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank Dr. Merima Alispahic for the professional input in MALDI-TOF MS analysis. We also thank Delfina Jandreski-Cvetkovic, Claudia Ibesich-Wawra, Attila Sandor and Vesna Stanesjalevic for their technical help. We appreciate the Electron Microscopy Facility, VBCF, Vienna, Austria for performing electron microscopy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.12.002.

References

[1] Nolan LK, Vaillancourt J-P, Barbieri NL, Logue CM. Colibacillosis. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. Diseases of Poultry. John Wiley & Sons, Inc.; 2020. p. 770–830.

[2] EFSA, Assessment of animal diseases caused by bacteria resistant to antimicrobials: Poultry. EFSA J. 2021;19(12):e07114.

[3] Ghunaim H, Abu-Madi MA, Kariyawasam S. Advances in vaccination against avian pathogenic *Escherichia coli* respiratory disease: potentials and limitations. Vet Microbiol 2014;172(1–2):13–22. https://doi.org/10.1016/j.vetmic.2014.04.019.

[4] Swelum AA, Elbestawy AR, El-Saadony MT, Hussein EOS, Alhotan R, Suliman GM, et al. Ways to minimize bacterial infections, with special reference to *Escherichia coli*, to cope with the first-week mortality in chicks: an updated overview. Poult Sci 2021;100(5). https://doi.org/10.1016/j.psj.2021.101039.

[5] Christensen H, Bachmeier J, Bisgaard M. New strategies to prevent and control avian pathogenic *Escherichia coli* (APEC). Avian Pathol 2021;50(5):370–81. https://doi.org/10.1080/03079457.2020.1845300.

[6] Kathayat D, Lokesh D, Ranjit S, Rajashekara G. Avian Pathogenic *Escherichia coli* (APEC): An Overview of Virulence and Pathogenesis Factors, Zoonotic Potential, and Control Strategies. Pathogens 2021;10(4). https://doi.org/10.3390/pathogens10040467.

[7] Gregersen RH, Christensen H, Ewers C, Bisgaard M. Impact of *Escherichia coli* vaccine on parent stock mortality, first week mortality of broilers and population diversity of *E. coli* in vaccinated flocks. Avian Pathol 2010;39(4):287–95. https://doi.org/10.1080/03079457.2010.495744.

[8] Landman WJM, van Eck JHH. The efficacy of inactivated *Escherichia coli* autogenous vaccines against the *E. coli* peritonitis syndrome in layers. Avian Pathol 2017;46(6):658–65. https://doi.org/10.1080/03079457.2017.1346231.

[9] Kromann S, Olsen RH, Bojesen AM, Jensen HE, Thofner I. Protective Potential of an Autogenous Vaccine in an Aerogenous Model of *Escherichia coli* Infection in Broiler Breeders. Vaccines (Basel) 2021;9(11). https://doi.org/10.3390/vaccines9111233.

[10] Li L, Thofner I, Christensen JP, Ronco T, Pedersen K, Olsen RH. Evaluation of the efficacy of an autogenous *Escherichia coli* vaccine in broiler breeders. Avian Pathol 2017;46(3):300–8. https://doi.org/10.1080/03079457.2016.1267857.

[11] Frommer A, Freidlin PJ, Bock RR, Leitner G, Chaffer M, Heller ED. Experimental vaccination of young chickens with a live, non-pathogenic strain of *Escherichia coli*. Avian Pathol 1994;23(3):425–33. https://doi.org/10.1080/03079459408419013.

[12] La Ragione RM, Woodward MJ, Kumar M, Rodenberg J, Fan H, Wales AD, et al. Efficacy of a live attenuated *Escherichia coli* O78:K80 vaccine in chickens and turkeys. Avian Dis 2013;57(2):273–9. https://doi.org/10.1637/10326-081512-Reg.1.

[13] Mombarg M, Bouzoubaa K, Andrews S, Vanimisetti HB, Rodenberg J, Karaca K. Safety and efficacy of an aroA-deleted live vaccine against avian colibacillosis in a multicenter field trial in broilers in Morocco. Avian Pathol 2014;43(3):276–81. https://doi.org/10.1080/03079457.2014.917760.

[14] Gharib AA, Hamouda AM, Abdel-Wahab AAM, Fawzy MF. Protective Efficacy of a Commercial Live Attenuated aroA mutant Vaccine Against Avian Pathogenic Escherichia coli Challenge in Broilers. Zagazig Veterinary Journal 2017;45(4):366–75. https://doi.org/10.21608/zvjz.2017.7867.

[15] Mohamed MA, Bakhit BM, Ibrahim AA, Saleh M. Evaluation of The Living Escherichia coli-O78 Deleted aroA Vaccine Against Homologous and Heterologous *E. coli* Challenge in Broiler Chickens. Journal of Advanced. Vet Res 2016;6(3):89–92.

[16] Koutsianos D, Gantelet H, Franzo G, Lecoupeur M, Thibault E, Cecchinato M, et al. An Assessment of the Level of Protection Against Colibacillosis Conferred by Several Autogenous and/or Commercial Vaccination Programs in Conventional Pullets upon Experimental Challenge. Vet Sci 2020;7(3). https://doi.org/10.3390/vetsci7030080.

[17] Kariyawasam S, Wilkie BN, Gyles CL. Construction, characterization, and evaluation of the vaccine potential of three genetically defined mutants of avian pathogenic Escherichia coli. Avian Dis 2004;48(2):287–99. https://doi.org/10.1637/7093.

[18] El-Mawgoud AIA, El-Nahass ES, Shany SAS, El-Sawah AA, Dahshan AM, Nasef SA, et al. Efficacy of Live Attenuated Vaccine and Commercially Available Lectin Against Avian Pathogenic *E. coli* Infection in Broiler Chickens. Vet Sci 2020;7(2). https://doi.org/10.3390/vetsci7020065.

[19] Nagano T, Kitahara R, Nagai S. An attenuated mutant of avian pathogenic Escherichia coli serovar O78: a possible live vaccine strain for prevention of avian colibacillosis. Microbiol Immunol 2012;56(9):605–12. https://doi.org/10.1111/j.1348-0421.2012.00482.x.

[20] OIE, Report of the meeting of the ad hoc group on prioritization of diseass for which vaccines could reduce antimicrobial use in animals, Paris, 21 – 23 April 2015 2015.

[21] Bhatia SS, Pillai SD. Ionizing Radiation Technologies for Vaccine Development - A Mini Review. Front Immunol 2022;13. https://doi.org/10.3389/fimmu.2022.845514.

[22] Tahergorabi R, Matak KE, Jaczynski J. Application of electron beam to inactivate Salmonella in food: Recent developments. Food Res Int 2012;45:685–94. https://doi.org/10.1016/j.foodres.2011.02.003.

[23] Sanakkayala N, Sokolovska A, Gulani J, Hogenesch H, Sriranganathan N, Boyle SM, et al. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant Brucella abortus RB51. Clin Diagn Lab Immunol 2005;12(12):1429–36. https://doi.org/10.1128/CDLI.12.12.1429-1436.2005.

[24] Magnani DM, Harms JS, Durward MA, Splitter GA. Nondividing but metabolically active gamma-irradiated Brucella melitensis is protective against virulent B. melitensis challenge in mice. Infect Immun 2009;77(11):5181–9. https://doi.org/10.1128/IAI.00231-09.

[25] Datta SK, Okamoto S, Hayashi T, Shin SS, Mihajlov I, Fermin A, et al. Vaccination with irradiated Listeria induces protective T cell immunity. Immunity 2006;25(1):143–52. https://doi.org/10.1016/j.immuni.2006.05.013.

[26] Bagheri S, Paudel S, Wijewardana V, Kangethe RT, Cattoli G, Hess M, et al. Production of interferon gamma and interleukin 17A in chicken T-cell subpopulations hallmarks the stimulation with live, irradiated and killed avian pathogenic Escherichia coli. Dev Comp Immunol 2022;133:. https://doi.org/10.1016/j.dci.2022.104408104408.

[27] Zloch A, Kuchling S, Hess M, Hess C. Influence of alternative husbandry systems on postmortem findings and prevalence of important bacteria and parasites in layers monitored from end of rearing until slaughter. Vet Rec 2018;182(12):350. https://doi.org/10.1136/vr.104632.

[28] Rezaee MS, Liebhart D, Hess C, Hess M, Paudel S. Bacterial Infection in Chicken Embryos and Consequences of Yolk Sac Constitution for Embryo Survival. Vet Pathol 2021;58(1):71–9. https://doi.org/10.1177/0300985820960127.

[29] Abdelhamid MK, Quijada NM, Dzieciol M, Hatfaludi T, Bilic I, Selberherr E, et al. Co-infection of Chicken Layers With Histomonas meleagridis and Avian Pathogenic Escherichia coli Is Associated With Dysbiosis, Cecal Colonization and Translocation of the Bacteria From the Gut Lumen. Front Microbiol 2020;11(1):. https://doi.org/10.3389/fmicb.2020.586437586437.
[30] Gaussmann B, Hess C, Grafl B, Kovacs M, Troxler S, Stessl B, et al. Escherichia coli isolates from femoral bone marrow of broilers exhibit diverse pheno- and genotypic characteristics that do not correlate with macroscopic lesions of bacterial chondronecrosis with osteomyelitis. Avian Pathol 2018;47 (3):271–80. https://doi.org/10.1080/03079457.2018.1440065.
[31] Alispahic M, Christensen H, Hess C, Razzazi-Fazeli E, Bisgaard M, Hess M. Identification of Gallibacterium species by matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry evaluated by multilocus sequence analysis. Int J Med Microbiol 2011;301(6):513–22. https://doi.org/10.1016/j.ijmm.2011.03.001.
[32] Paudel S, Fink D, Abdelhamid MK, Zoggeler A, Liebhart D, Hess M, et al. Aerosol is the optimal route of respiratory tract infection to induce pathological lesions of colibacillosis by a lux-tagged avian pathogenic Escherichia coli in chickens. Avian Pathol 2021;50(5):417–26. https://doi.org/10.1080/03079457.2021.1978392.
[33] Paudel S, Hess M, Hess C. Coinfection of Avibacterium paragallinarum and Gallibacterium anatis in Specific-Pathogen-Free Chickens Complicates Clinical Signs of Infectious Coryza, Which Can Be Prevented by Vaccination. Avian Dis 2017;61(1):55–63. https://doi.org/10.1637/11481-081016-Reg.
[34] R-Core-Team, A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. 2021.
[35] Christensen, RHB. ordinal: Regression Models for Ordinal Data. R Packag. version 2019.12-10 2019, https://CRAN.R-project.org/package=ordinal. 2019.
[36] Fox J, Weisberg S. An R Companion to Applied Regression. Third ed. Thousand Oaks: California SAGE Publications, Inc; 2019.
[37] Lenth, RV, Buerkner P, Herve M, Love J, Miguez F, Riebl H, et al. emmeans: Estimated Marginal Means, aka Least-Squares Means. R Packag. version 1.5.5-1 2021, https://CRAN.R-project.org/package=emmeans. 2022.
[38] Bhatia, SS, Pillai SD. A Comparative Analysis of the Metabolomic Response of Electron Beam Inactivated E. coli O26:H11 and Salmonella Typhimurium ATCC 13311. Front Microbiol. 2019;10:694. DOI: 10.3389/fmicb.2019.00694.
[39] Praveen C, Bhatia SS, Alaniz RC, Droleskey RE, Cohen ND, Jesudhasan PR, et al. Assessment of microbiological correlates and immunostimulatory potential of electron beam inactivated metabolically active yet non culturable (MAyNC) Salmonella Typhimurium. PLoS One 2021;16(4):e0243417. https://doi.org/10.1371/journal.pone.0243417.
[40] Hieke AC, Pillai SD. Escherichia coli Cells Exposed to Lethal Doses of Electron Beam Irradiation Retain Their Ability to Propagate Bacteriophages and Are Metabolically Active. Front Microbiol 2018;9:2138. https://doi.org/10.3389/fmicb.2018.02138.
[41] Kato M, Futenma A. Expression of the lacZ gene in Escherichia coli irradiated with gamma rays. J Radiat Res Appl Sci 2014;7(4):568–71. https://doi.org/10.1016/j.jrras.2014.09.008.
[42] Ko E, Jeong S, Jwa MY, Kim AR, Ha YE, Kim SK, et al. Immune Responses to Irradiated Pneumococcal Whole Cell Vaccine. Vaccines (Basel) 2021;9(4). https://doi.org/10.3390/vaccines9040405.
[43] Tracz DM, McCorrister SJ, Westmacott GR, Corbett CR. Effect of gamma radiation on the identification of bacterial pathogens by MALDI-TOF MS. J Microbiol Methods 2013;92(2):132–4. https://doi.org/10.1016/j.mimet.2012.11.013.
[44] Williams TL, Andrzejewski D, Lay JO, Musser SM. Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. J Am Soc Mass Spectrom 2003;14 (4):342–51. https://doi.org/10.1016/S1044-0305(03)00065-5.
[45] Melamed D, Leitner G, Heller ED. A vaccine against avian colibacillosis based on ultrasonic inactivation of Escherichia coli. Avian Dis 1991;35(1):17–22.
[46] Holden KM, Browning GF, Noormohammadi AH, Markham P, Marenda MS. Avian pathogenic Escherichia coli DeltatonB mutants are safe and protective live-attenuated vaccine candidates. Vet Microbiol 2014;173(3–4):289–98. https://doi.org/10.1016/j.vetmic.2014.07.028.
[47] Filho TF, Favaro Jr C, Ingberman M, Beirao BC, Inoue A, Gomes L, et al. Effect of spray Escherichia coli vaccine on the immunity of poultry. Avian Dis 2013;57 (3):671–6. https://doi.org/10.1637/10456-112612-ResNote.1.
[48] Hellfritzsch M, Scherliess R. Mucosal Vaccination via the Respiratory Tract. Pharmaceutics 2019;11(8). https://doi.org/10.3390/pharmaceutics11080375.
[49] Li Y, Wang Z, Liu X, Tang J, Peng B, Wei Y. X-ray Irradiated Vaccine Confers protection against Pneumonia caused by Pseudomonas aeruginosa. Sci Rep 2016;6:18823. https://doi.org/10.1038/srep18823.
[50] Fertey J, Thoma M, Beckmann J, Bayer L, Finkensieper J, Reisshauer S, et al. Automated application of low energy electron irradiation enables inactivation of pathogen- and cell-containing liquids in biomedical research and production facilities. Sci Rep 2020;10(1):12786. https://doi.org/10.1038/s41598-020-69347-7.