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Respiratory health of broilers following chronic exposure to airborne endotoxin

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ABSTRACT

In and around poultry farms, high concentrations of endotoxins are found that have a negative impact on the health of farmers and local residents. However, little is known about the effects of chronic exposure to endotoxins on the health of poultry. The aim of this study was to identify effects of chronic exposure to airborne endotoxins (*E. coli* LPS) on the immune system, respiratory tract, disease susceptibility and welfare of broilers. Effects of high (HE) and low endotoxin (LE) concentrations on natural antibody titers (NAb), performance and behavior of broilers were determined. After treatment with a respiratory virus infection, infectious bronchitis virus (IBV), mRNA expression of cytokines and Toll-like receptor (TLR) 4 in the lung, tracheal ciliary activity and lesions in the respiratory tract were determined. Endotoxin affected the immune system and respiratory tract, where HE broilers tended to have lower IgM NAb binding Phosphorylcholine-conjugated to Bovine Serum Albumin, and higher interferon (IFN)-α mRNA expression and more lesions in the nasal tissue compared to LE broilers. Furthermore, HE broilers had higher TLR4 mRNA expression compared to LE broilers. However, endotoxin did not affect NAb levels binding Keyhole Limpet Hemocyanin, IFN-β and interleukin-10 mRNA expression, IBV replication or lesions in the lung and trachea. HE and LE broilers further had similar body weight, but HE broilers showed numerically more passive behavior compared to LE broilers. In conclusion, chronic exposure to high airborne endotoxin concentrations affects components of the immune system and respiratory tract in broilers and could therefore influence disease susceptibility.

1. Introduction

The air in and around livestock farms contains high concentrations of endotoxins (Maassen et al., 2016; Seedorf et al., 1998). Endotoxins are cell-wall components of gram-negative bacteria, also known as lipopolysaccharides (LPS). High levels of LPS can be present in animal debris such as feathers, hair, skin flakes and feces (Cambra-López et al., 2011). Endotoxins are large molecules and can bind to different types of residues and dust particles, and can thus spread into the environment. They activate the innate immune system when LPS binds to LPS-binding protein (LBP) and subsequently binds to CD14 which is then recognized by Toll-like receptor (TLR) 4. Binding of LPS to the CD14/TLR4 complex activates macrophages and triggers pro-inflammatory cytokine production (Heumann and Roger, 2002). Thus, when humans or animals are regularly exposed to endotoxins this may affect their immune reactivity and health.

Farmers are frequently exposed to endotoxins and the majority of studies show a negative impact on health outcomes in farmers (Douglas et al., 2018). Endotoxins can cause impaired lung function and respiratory symptoms such as coughing, wheezing, chest tightness, shortness of breath (Donham et al., 2000; Eduard et al., 2009; Vogelzang et al., 1998; Zejda et al., 1994) and increase the risk of lung diseases, such as non-atopic asthma (Eduard et al., 2004) and chronic obstructive pulmonary diseases (COPD) (Eduard et al., 2009; Zejda et al., 1994). Living near livestock farms with high concentrations of endotoxin can also affect the health of local residents as they had reduced lung function and increased prevalence of asthma symptoms (Hoopmann et al., 2006; Schinasi et al., 2011). In the Netherlands, however, people living near

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livestock farms had less asthma and less allergies, but they did show reduced lung function and had higher risk of getting pneumonia (Smit et al., 2017, 2014). These findings indicate that both farmers and local residents can experience a negative impact on their health due to endotoxin exposure.

Poultry houses were shown to have the highest concentration of endotoxin in comparison to cattle and pig houses (Seedorf et al., 1998), and poultry farms are known for their high endotoxin load towards the environment (Maassen et al., 2016). Furthermore, poultry farmers had lower lung function than other farmers and workers (Radon et al., 2001; Zuskin et al., 1995), and living near poultry farms was also associated with increased risk of pneumonia (Smit et al., 2017). Yet, most studies to date have focused on identifying effects of endotoxin exposure in humans, but little is known about the effects of chronic exposure to airborne endotoxin on poultry health.

Previous studies have shown that endotoxin can affect poultry health. LPS was shown to induce pulmonary hypertension in broilers housed under commercial conditions (Lorenzoni and Wideman, 2008). Furthermore, LPS has immunomodulatory effects in poultry, where it decreased (Maldonado et al., 2005; Parmentier et al., 2006, 2004) or increased (Parmentier et al., 2008, 2006) the specific antibody response in poultry depending on the concentration and mode of application. LPS further increased mRNA expression of interleukin-6 (IL-6), inducible nitric oxide synthase and nuclear factor-kappa-B (Kaiser et al., 2010; Yang et al., 2008). However, all these studies focused on identifying effects of intratracheal, intravenous, intraperitoneal or subcutaneous LPS administration (often only short exposure and a high dose) and not on effects of chronic exposure to airborne LPS. Only one study to date focused on the relation between endotoxin concentrations in a farm and chicken immunity, where laying hens from a farm with high endotoxin concentrations showed lower IFN-γ production by T cells and less B cells in their blood compared to laying hens from a farm with low endotoxin concentrations, but no differences in IFN-γ production or proportion of lymphocyte subpopulations were found for broilers housed in high or low endotoxin concentrations (Roque et al., 2015). Thus, chronic exposure to airborne endotoxins could affect the chicken immune system and thereby potentially also disease susceptibility and welfare.

The aim of this study was to identify effects of chronic exposure to airborne endotoxins (*E. coli* LPS) on the immune system, respiratory tract, disease susceptibility and welfare of broilers. We identified natural antibody (NAb) titers towards Keyhole Limpet Hemocyanin (KLH) and Phosphorylcholine-conjugated to Bovine Serum Albumin (PC-BSA), performance and behavior of broilers housed in high or low endotoxin environments. Infection with virulent or attenuated infectious bronchitis virus (IBV) was used as an infection model to explore differences in disease susceptibility, after which we identified mRNA expression of cytokines and TLR4, tracheal ciliary activity and lesions in the respiratory tract. NAb titers were measured as natural antibodies play an essential role in both innate and adaptive immunity, for example by maintaining homeostasis, increasing disease resistance and linking the two types of immunity (Berghof et al., 2019; Panda and Ding, 2015), and could therefore play a role in broiler health. Performance and behavior were measured to assess broiler welfare. Expression of interferon-alpha (IFN-α) and IFN-β were identified as they play a crucial role in anti-viral responses (Zhang et al., 2017). Expression of IL-10 and TLR4 were identified as they play crucial roles in LPS signaling (Keestra and van Putten, 2008; Wu et al., 2016). Furthermore, tracheal ciliary activity and lesions in the respiratory tract were measured to assess the response to IBV in relation to the different endotoxin environments. We hypothesized that chronic exposure to high endotoxin concentrations would negatively affect the immune system, respiratory tract and health of broilers after a respiratory infection.

2. Materials and methods

2.1. Ethics statement

The experiment was approved by the Central Authority for Scientific Procedures on Animals in accordance with the Dutch regulations (no: AVD401002016578).

2.2. Animals and housing

The experiment was conducted with 60 one-day-old Ross 308 broilers from a commercial breeder. Broilers were equally divided over two identical climate-controlled rooms (2 m × 3.3 m each) with a 50/50 male/female distribution in each room and with feed and water provided ad libitum. No antibiotics were given during the experiment. Floor pens had wood shavings provided as bedding. Bedding was refreshed every week during the first 3 weeks and twice a week during the last 2 weeks of the experiment to ensure that no additional (uncontrolled levels of) endotoxins from manure and feathers were present.

From 1 to 34 days of age, 30 broilers were housed in low endotoxin concentrations (LE) and 30 broilers in high endotoxin concentrations (HE). Endotoxin was lipopolysaccharides (LPS) from *Escherichia coli* 055:B5 purified by phenol extraction (Sigma Aldrich®, St. Louis, USA). Purified endotoxin was added to PM_{10} (particulate matter with aerodynamic diameter < 10 μm) in a concentration of 1000 EU/mg. In the HE room, 1.0 g solution per m^3 of incoming air was atomized per min during daytime (6.00 am to 10.00 pm) and 0.4 g solution per m^3 of incoming air was atomized per min during nighttime (10.00 pm to 6.00 am) to simulate the pattern under commercial circumstances (Aarnink et al., 2011). From day 19 to 34, endotoxins were dissolved and atomized without adding PM_{10}, as adding PM_{10} after day 19 resulted in unequal distribution of endotoxins.

At 34 days of age, all broilers were transported to another research facility in order to investigate the effect of endotoxin exposure on susceptibility to infection. All broilers were housed in a biosafety level 2 room and in a similar way as up to day 34, but without additional endotoxin exposure. Broilers from the HE and LE rooms were randomly divided into three groups each with 10 broilers per group (including 5 males and 5 females). One group per treatment was euthanized immediately after arrival by cervical dislocation and sampled (i.e., control). One group per treatment was inoculated intraocular (max. 0.1 ml of fluid) with the virulent Infectious Bronchitis Virus, strain M41 ($10^{4.3}$ EID_{50}/ml) (i.e. IBV challenged) and one group per treatment was vaccinated intraocular with an attenuated live IBV-spray vaccine (Nobilis® IB MA5, MSD Animal Health) containing the attenuated IBV MA5 strain ($10^{3.5}$ EID_{50}/ml) (i.e. IBV vaccinated). IBV was chosen as it is known to cause an infection of the respiratory tract and especially of the trachea. Attenuated vaccine strains are used to elicit an immune response for protection, but are known to also induce mild tracheal changes and can increase disease susceptibility for colibacillosis (Matthijs et al., 2003). Three days post challenge (i.e. 37 days of age) all remaining broilers were euthanized and tissues were sampled.

2.3. Measurement of PM10 and endotoxin

During the experiment, PM_{10} and endotoxin concentrations were measured. Continuous measurements of PM_{10} concentration were carried out using a light-scattering device (DustTrak Aerosol Monitor, model 8520, TSI Inc., Shoreview, USA) (Winkel et al., 2015). In addition, filter samples were taken at 14, 21, 28, and 30 days of age and stored at −20 °C until further analysis. Endotoxin concentration was analyzed with the quantitative Limulus Amebocyte Lysate (LAL) assay (BioWhittaker Inc., Walkersville, MD, USA). A sample was mixed with the LAL in the test kit and incubated at 37 °C (±1 °C) for 10 min. A substrate sample was then mixed with the LAL sample and incubated at 37 °C (±1 °C) for another 6 min, after which the reaction was stopped.

Extinctions were measured with a spectrophotometer at 405–410 nm. Because the absorption is linearly related to the amount of endotoxin in the sample, the endotoxin concentration can be determined with a standard curve (Spaan et al., 2006).

2.4. Performance and behavior

Body weight and feed intake were determined weekly by weighing the individual broilers and feed supply/leftover. Behavioral observations were performed via scan sampling at 3 and 5 weeks of age during three separate sessions on 1 day: in the morning (8:30–9.30 h), in the beginning of the afternoon (12:00–13:00 h) and at the end of the afternoon (16:00–17:00 h). Each session consisted of 5 scans of 10 min with 3 observations per room, alternating between the HE and LE rooms. Each observation consisted of counting the number of broilers that performed a certain behavior during five successive scans. Behaviors were scored in three categories: active, passive and other. Active behaviors included: eating, drinking, foraging, dustbathing, comfort (preening, stretching, yawning), feather pecking, object pecking, aggressive behavior, standing (active head up, eyes open). Passive behaviors included: standing (passive, head between shoulders), sitting, huddling, sleeping (head in feathers). Other behaviors included behavior other than above listed behaviors. All behavioral observations were performed by the same observer.

2.5. Natural antibody titers

Blood was collected at 34 days of age, before transportation by jugular vein puncture and collected in serum separating tubes (Greiner Bio-one, Alphen aan den Rijn, The Netherlands). Samples were kept at room temperature until incubation for 1 h at 37 °C. Samples were then centrifuged at 5251 ×*g* for 12 min at −20 °C and serum was stored at −80 °C until further analysis. IgM and IgG natural antibody titers binding keyhole limpet hemocyanin (KLH, Sigma-Aldrich, St. Louis, MO, USA) or Phosphorylcholine-conjugated to Bovine Serum Albumin (PC-BSA, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were determined by a two-step indirect enzyme-linked immunosorbent assay (ELISA) as described in Parmentier et al. (2008). Each absorbance was expressed relatively to the absorbance of a standard positive control serum sample, and antibody titers were expressed as log2 values of dilutions that gave extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive serum present on every microtiter plate.

2.6. Lung, bronchia and beak sampling

Broilers were anaesthetized with Zoletil® 100, killed by cervical dislocation and necropsied for the collection of lung tissue (cross sections of the mid part of the left lung), trachea (3 sections at cranial, mid and caudal location) and beaks were fixated in 4% (v:v) buffered formalin for histopathological analyses. Two pieces of right lung tissues were snap-frozen in liquid nitrogen and stored at −70 °C until RNA extraction. Total RNA was extracted from the frozen tissue samples as described by Rebel et al. (2005) and Cornelissen et al. (2009).

2.7. IBV detection by real-time quantitative PCR

For the quantification of IBV viral load, RNA was isolated from lung tissue using the Direct-zol ™ RNA MiniPrep (Zymo Research) according to manufacturer's instructions and cDNA was synthesized using Quantitect ® Reverse Transcription Kit (Qiagen). A qRT-PCR was performed with the Applied Biosystems 7500/7500 standard™. The reaction mixture (20 µl) contained 12.5µl of SYBR®Green PCR Mastermix, 15 µM of each primer (Fw: 5′- GCTTTTGAGCCTAGCGTT −3′, Rev.: 5′-GCCATGTTGTCACTGTCTATTG −3′) according to Callison et al. (2006). To quantify the viral load a standard curve was established from an egg titrated IBV strain M41 virus culture.

2.8. Tracheal ciliary activity

To evaluate tracheal ciliostasis, three sections of the upper, middle, and lower parts of the trachea (nine rings per bird) were analyzed, as described by Geerligs et al. (2011). Briefly, trachea rings were examined by light microscopy to determine the ciliary movement in the tracheal epithelial cells. A score of 0 to 5 was given, 0 = 100% movement, 1 = 75% to 100%, 2 = 50% to 75%, 3 = 25% to 50%, 4 = 0 to 25% and 5 = 0% movement.

2.9. Histopathology

Formalin-fixed beak, trachea and lung sections (5 µm thick) were stained with haematoxylin and eosin (HE staining) and were examined by light microscopy for pathological and histological disorders. The examination encompassed the extent of lesions of the respiratory tract in each slide, i.e. presence of focal or diffuse alterations with interstitial or catarrhal pneumonia or atelectasis, the extent of infiltration of alveolar septae with mononuclear cells and the extent of infiltration of mononuclear cells in the perivascular/peribronchiolar area. A histological score from 0 to 4 was given, 0 = no specific findings with few mononuclear immune cells in the mucosa, 1 = mild increase of immune cells with few, focal lymphoid aggregates, 2 = moderate inflammation, 3 = moderate to severe inflammation and 4 = severe diffuse and extended mucosa inflammation manifestation was used to describe the severity of changes. To compare the histological findings between groups, the scores from each slide were added to an overall score and divided by the amount of slides to obtain the mean, which could add up to a maximum of 4.

2.10. Cytokine and TLR4 mRNA expression

For the quantification of cytokine and TLR4 mRNA, cDNA was made of lung tissue using random hexamer primers and reverse transcriptase. The PCR was employed with on-line detection of the PCR reaction using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) in an ABI 7500 Real-Time PCR system (Applied Biosystems). Post amplification melting curves with SYBR Green Dye revealed one peak. Primers were as described in Table 1, annealing temperatures were 59 °C. Relative expression values were normalized against 28S rRNA (Cornelissen et al., 2009). For the quantification, a standard curve of the plasmid with the insert of the cytokine-encoding gene of interest, constructed in pGEM-T easy was used (Promega Benelux b.v., Leiden, The Netherlands). Results were presented as mean fold induction in samples from 10 chickens for each time point.

2.11. Statistical analysis

SAS Software version 9.4 was used for statistical analysis (SAS Institute). Linear mixed models for endotoxin effects on body weight consisted of fixed effects of endotoxin (i.e. HE or LE) * age, endotoxin,

Table 1
Primers used for Real Time quantitative PCR.

Target[a]	Primer sequence (5′-3′)	
	Forward	Reverse
IFN-α	TTCAGCTGCCTCCACACCTT	TTGTGGATGTGCAGGAACCA
IFN-β	CAGCTCTCACCACCACCTTCTC	GGAGGTGGAGCCGTATTCTG
IL-10	CGCTGTCACCGCTTCTTCA	TCCCGTTCTCATCCATCTTCTC
TLR-4	TGCATGAGCTCTGTGGTTGTC	AGCCCGTTCATCCTCATATCTC
28S	CAAGTCCTTCTGATCGAG	TCAACTTTCCCTTACGGTAC

[a] Interferon-α (IFN-α), Interferon- β (IFN-β), Interleukin-10 (IL-10) and Toll-like 4 Receptor (TLR4).

age and sex. The random effect consisted of a repeated statement for age with chicken ID as subject and an unstructured covariance structure. Linear mixed models for endotoxin effects on NAb titers consisted of fixed effects endotoxin and sex. Linear mixed models were further used to analyze endotoxin effects on histological nasal scores and consisted of fixed effects of endotoxin * treatment (i.e. control, IBV challenge or IBV vaccination), endotoxin, treatment and sex. The model residuals were visually examined for normality. Post hoc pairwise comparisons were corrected by Tukey-Kramer adjustment. Data from IBV RNA, ciliostasis scores, histological lung and trachea scores, and relative mRNA expression of IFN-α, IFN-β, IL-10 and TLR4 did not meet model assumptions of normality and therefore we used Kruskal Wallis tests to identify endotoxin * treatment and endotoxin effects. Post hoc comparisons were made with Dwass, Steel, Critchlow-Fligner method to correct for multiple comparisons. Data from one broiler (HE broiler that received IBV vaccination) that had a very low value for 28S quantity was considered as an outlier and therefore excluded from further analyses for cytokine and TLR4 mRNA expression. All data are presented as mean ± standard error (SE). Feed intake and behavioral data were not statistically analyzed as only two rooms were used with one pen each and pen was the experimental unit for these measurements since feed intake was measured at pen level and individuals within a pen can influence each other's behavior ($n = 1$). For the other measurements we considered individual broilers as the experimental unit ($n = 30$ for body weight and NAb titers and $n = 10$ for ciliostasis scores, histopathology and mRNA expression).

3. Results

3.1. Endotoxin concentration, performance and behavior

The median endotoxin concentrations were 119 EU/m3 in the low endotoxin (LE) environment and 10,802 EU/m3 in the high endotoxin (HE) environment (Fig. 1), indicating contrasts in endotoxin concentrations. Body weight did not differ between LE and HE broilers at any age (average body weight at 34 days of age: LE = 2093.3 g HE = 2137.7 g). Behaviors were summarized in average percentage of broilers showing active and passive behaviors (Fig. 2). HE broilers showed numerically more passive behavior at 5 weeks of age compared to LE broilers, especially in the afternoon.

3.2. Natural antibodies

No significant endotoxin effect was found on IgM and IgG antibody titers to keyhole limpet hemocyanin (KLH, Fig. 3A & B) or on IgG antibody titers to phosphorylcholine conjugated to bovine serum albumin (PC-BSA, Fig. 3D). Yet, endotoxin tended to affect IgM titers to PC-BSA ($F_{1,56} = 2.89$, $P < 0.1$, Fig. 3C), with LE broilers having higher IgM titers compared to HE broilers.

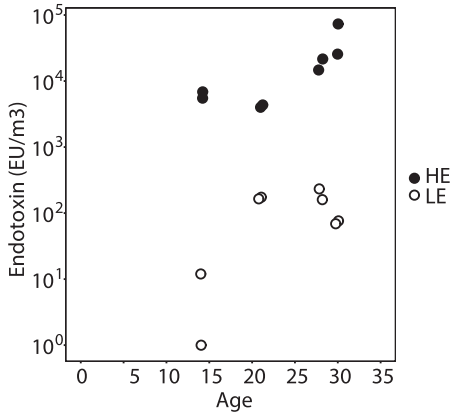

Fig. 1. Endotoxin concentration (EU/m^3) at 14, 21, 28 and 30 days of age in the high endotoxin (HE) and low endotoxin (LE) environment.

3.3. IBV detection

HE and LE broilers were either challenged with IBV, received an IBV vaccination or did not receive treatment (control) at 34 days of age. A significant endotoxin * treatment interaction was found on quantity of viral RNA in lung tissue ($\chi^2 = 28.9$, df = 3, $P < 0.01$), but HE and LE broilers did not differ in quantity of viral RNA when comparing them within IBV challenged and IBV vaccinated groups. When comparing within HE and LE broilers, IBV challenged broilers had higher quantity of viral RNA compared to IBV vaccinated broilers ($P < 0.01$).

3.4. Tracheal ciliary activity

Three days after the treatment (at 37 days of age), broilers were euthanized and tracheal ciliary activity was measured (i.e. ciliostasis score). A significant endotoxin * treatment interaction was found on ciliostasis score ($\chi^2 = 54.4$, df = 5, $P < 0.01$), but HE and LE broilers did not differ in tracheal ciliary activity when comparing them within control, IBV challenged and IBV vaccinated groups. When comparing within HE and LE broilers, IBV challenged broilers had higher ciliostasis scores (i.e. lower tracheal ciliary activity) compared to IBV vaccinated broilers ($P < 0.01$) and compared to control broilers ($P < 0.01$). IBV vaccinated broilers also had higher ciliostasis scores compared to control broilers ($P < 0.01$). Overall, HE and LE broilers did not differ in tracheal ciliary activity (Fig. 4).

3.5. Histopathology

The lesions and disturbances of the lung, trachea and beak (nasal epithelial) were scored. A significant endotoxin * treatment interaction was found on lung scores ($\chi^2 = 43.2$, df = 5, $P < 0.01$) and trachea scores ($\chi^2 = 40.2$, df = 5, $P < 0.01$), but HE and LE broilers did not differ in lesions in the lung and trachea when comparing them within control, IBV challenged or IBV vaccinated groups (Fig. 5A & B). IBV challenged broilers had more lesions in the lung and tended to have more lesions in the trachea compared to IBV vaccinated broilers ($P < 0.05$ and $P < 0.1$, respectively) and more lesions in the lung and trachea compared to control broilers ($P < 0.01$). IBV vaccinated broilers also had more lesions in the lung and trachea compared to control broilers ($P < 0.05$). Overall, HE and LE broilers did not differ in lesions and disturbances of the lung and trachea. A significant endotoxin * treatment interaction was found on nasal scores ($F_{5,52} = 37.85$, $P < 0.01$), within the IBV vaccinated group, broilers housed in the LE environment tended to have lower nasal scores (i.e. less lesions) compared to those housed in the HE environment (Fig. 5C). Control broilers had less lesions in the nasal tissue compared to IBV challenged and IBV vaccinated broilers ($P < 0.01$), but IBV challenged and vaccinated broilers did not differ in nasal lesions.

3.6. Cytokines and TLR4 mRNA expression in lungs

The relative mRNA expression of IFN-α, IFN-β, IL-10 and TLR4 were analyzed. A significant endotoxin * treatment interaction was found on IFN-α ($\chi^2 = 17.94$, df = 5, $P < 0.01$), IFN-β ($\chi^2 = 11.71$, df = 5, $P < 0.05$) and TLR4 ($\chi^2 = 12.47$, df = 5, $P < 0.05$), but HE and LE broilers did not differ in mRNA expression when comparing them within control, IBV challenged or IBV vaccinated groups (Fig. 6A, B & D). Within the HE broilers, IBV challenged broilers had higher IFN-α mRNA expression compared to control broilers ($P < 0.05$). For IFN-β and TLR4, no significant differences were found between endotoxin * treatment groups after correction for multiple comparisons. However, when comparing HE to LE broilers, irrespective of treatment, HE broilers tended to have higher IFN-α mRNA expression ($\chi^2 = 3.22$, df = 1, $P < 0.1$) and had higher TLR4 mRNA expression compared to LE broilers ($\chi^2 = 8.47$, df =

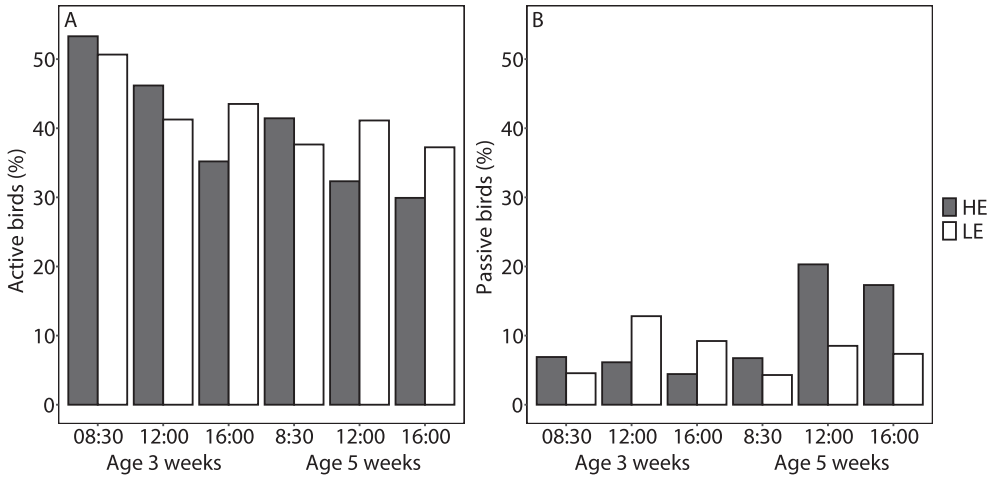

Fig. 2. Percentage of broilers showing A) active and B) passive behavior at 3 and 5 weeks of age in the morning (8:30–9.30 h), in the beginning of the afternoon (12.00–13.00 h) and at the end of the afternoon (16.00–17.00 h) for broilers in the high endotoxin (HE) or low endotoxin (LE) environment.

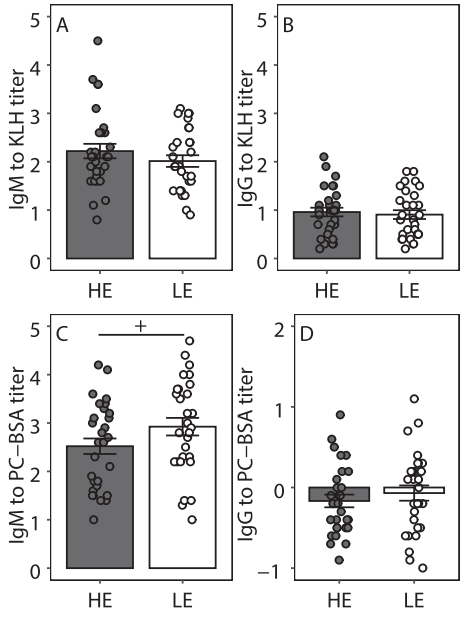

Fig. 3. Natural antibodies titers of IgM (A and C) and IgG (B and D) to keyhole limpet hemocyanin (KLH, A and B) and phosphorylcholine conjugated to bovine serum albumin (PC-BSA, C and D) at 34 days of age for broilers in the high endotoxin (HE) or low endotoxin (LE) environment ($n = 30$ broilers per endotoxin group). + denotes tendencies ($P < 0.1$).

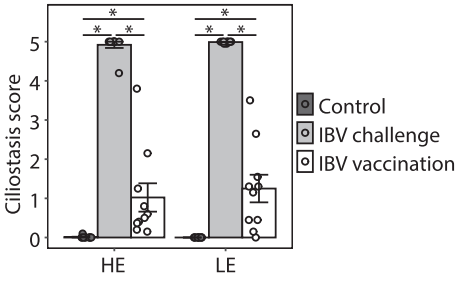

Fig. 4. Ciliostasis score (i.e. tracheal ciliary activity, with 0 = 100% movement to 5 = 0% movement) for broilers in the high endotoxin (HE) or low endotoxin (LE) environment and exposed to control treatment, IBV (Infectious Bronchitis Virus) challenge or IBV vaccination ($n = 10$ broilers per endotoxin * treatment group). * denotes significant differences ($P < 0.05$).

1, P < 0.01). When comparing the different treatment groups, IBV challenged broilers had higher IFN-α ($\chi^2 = 14.70$, df = 2, P < 0.01) and IFN-β ($\chi^2 = 9.45$, df = 2, P < 0.01) mRNA expression compared to both IBV vaccinated and control broilers (P < 0.01 and P < 0.05, respectively). For IL-10 no significant effects of endotoxin * treatment interaction or endotoxin were found on mRNA expression (Fig. 6C).

4. Discussion

The objective of the current study was to identify effects of chronic exposure to airborne endotoxins (*E. coli* LPS) on the immune system, respiratory tract, disease susceptibility and welfare of broilers. To summarize, endotoxin exposure affected immune parameters and the respiratory tract, where broilers housed in the HE environment tended to have lower IgM NAb binding PC-BSA, but higher IFN-α mRNA expression in the lung and more lesions in the nasal tissue after IBV vaccination compared to broilers housed in the LE environment. Furthermore, HE broilers had higher TLR4 mRNA expression in the lung and showed numerically more passive behavior at 5 weeks of age compared to LE broilers. However, HE and LE broilers had similar body weight and endotoxin exposure did not clearly affect NAb binding KLH, cytokines (IL-10, INF-β) mRNA expression in the lung, susceptibility to IBV, lesions in the lung and trachea.

In poultry farms, concentrations of airborne endotoxins ranging between 240 and 13,400 EU/m^3 have been reported (Douwes, 1998). The *E. coli* LPS concentration in the high endotoxin environment of the current experiment reached approximately three to twelve times the reported averages of 880 and 3814 EU/m3 for endotoxin concentrations measured in broiler houses in the Netherlands (Seedorf et al., 1998; Spaan et al., 2006). Thus, we were able to create endotoxin concentrations in the HE environment that were in the higher range of what has been found previously in commercial broiler farms. Furthermore, infectious bronchitis viral RNA was present in both IBV challenged and vaccinated broilers, and IBV challenged broilers had more viral RNA in the lungs and lower tracheal ciliary activity compared to IBV vaccinated and control broilers. Thus, the IBV challenge and vaccination were successful.

Chronic exposure to high endotoxin (*E. coli* LPS) concentrations did not affect NAb binding KLH, but did influence NAb binding PC-BSA. LE broilers tended to have higher IgM binding PC-BSA compared to HE broilers, although no effect was found on IgG binding PC-BSA. PC is a 'danger' related antigen and might even be considered as a self-antigen

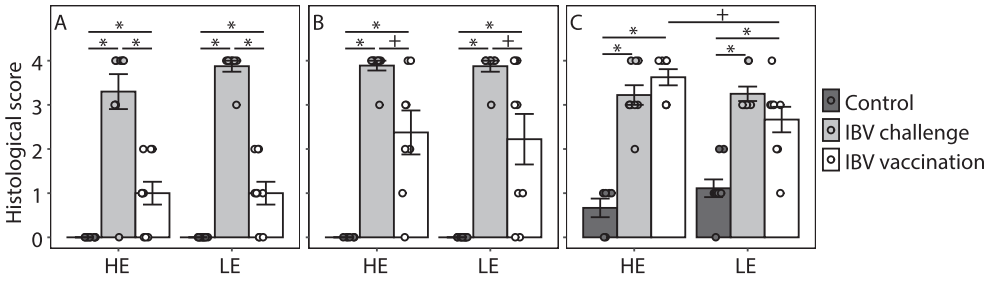

Fig. 5. Histology score (with 0 = no specific findings to 4 = severe diffuse and extended mucosa inflammation) of A) lung, B) trachea and C) nasal tissue for broilers in the high endotoxin (HE) or low endotoxin (LE) environment and exposed to control treatment, IBV (Infectious Bronchitis Virus) challenge or IBV vaccination (n = 10 broilers per endotoxin * treatment group). + denotes tendencies ($P < 0.1$) and * denotes significant differences ($P < 0.05$).

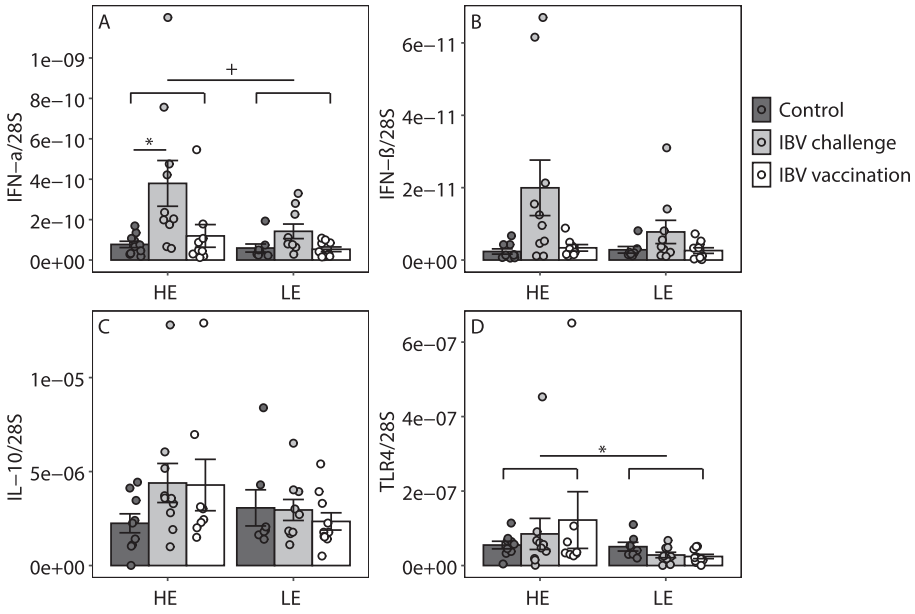

Fig. 6. Relative expression levels of cytokines A) Interferon-α (IFN-α), B) IFN-β and C) Interleukin-10 (IL-10) and of D) Toll-like 4 Receptor (TLR4) for lungs of broilers in the high endotoxin (HE) or low endotoxin (LE) environment and exposed to control treatment, IBV (Infectious Bronchitis Virus) challenge or IBV vaccination (n = 10 broilers per endotoxin * treatment group). Absolute mRNA levels were normalized to the corresponding mRNA levels of 28S. + denotes tendencies ($P < 0.1$) and * denotes significant differences ($P < 0.05$).

(Elkon and Silverman, 2012), therefore this result could indicate that endotoxin did affect natural autoantibody (NAAb) binding PC-BSA, i.e. NAb binding self-antigens. Previous studies support these findings, showing that intratracheal LPS challenge did not affect NAb levels to rabbit γ-globulin (Parmentier et al., 2006), but did affect NAAb levels to actin and thyroglobuline in laying hens (Berghof et al., 2010). Although effects of intratracheal LPS challenge on NAb levels to KLH were also reported in laying hens (Berghof et al., 2010). Thus, previous findings with regard to effects of endotoxin on NAb are inconsistent, potentially because of different types of antigens used, genetic background, administration route or frequency of exposure to endotoxin. Our data suggest that levels of NAb can be reduced by chronic exposure to high airborne endotoxin (*E. coli* LPS) concentrations. Since NAb play an essential role in both innate and adaptive immunity (Panda and Ding, 2015), modulation of NAb levels by airborne endotoxin may affect immunity and therefore disease susceptibility in broilers.

Chronic exposure to high endotoxin (*E. coli* LPS) concentrations did not influence IL-10 and INF-β mRNA expression in lung tissue, but resulted in higher expression of IFN-α and TLR4 mRNA in lung tissue 3 days post IBV infection. HE broilers tended to have higher IFN-α mRNA expression compared to LE broilers, suggesting a higher inflammatory response to the IBV infection. Previous studies found that treatment with LPS increased expression of IFN-α and IFN-β in chicken lung epithelial cells (Esnault et al., 2011) and increased expression of IFN-α and IL-10 in chicken spleens (St. Paul et al., 2011), but 1 day post treatment these effects were no longer present. Furthermore, treatment with LPS did not affect expression of IFN-α or IFN-β in embryonated chicken eggs between 4 h and 16 h post treatment (Sharma et al., 2020). Thus, previous findings with regard to effects of endotoxin on expression of IFN-α, IFN-β and IL-10 are inconsistent, potentially because of different types of organs collected for gene expression, different batches or origin of endotoxin, administration route and frequency of exposure to endotoxin. In the present study, chronic airborne endotoxin (*E. coli* LPS) exposure tended to affect IFN-α but not IFN-β or IL-10 expression. However, HE broilers had higher TLR4 mRNA expression compared to LE broilers and tended to have more lesions in the nasal tissue compared to LE broilers, but only within the IBV vaccinated group. Similarly, mice exposed to poultry barn air for 30 or 60 days or intranasally challenged with LPS or a combination of these had higher TLR4 mRNA expression and more damage in the lungs compared to control mice (Kaur and Sethi, 2020). Broilers intra-peritoneally injected with LPS showed higher TLR4 protein expression and had more damage in the lungs and trachea compared to broilers injected with saline, although no differences were found 3 days after injection (Ansari et al., 2016). Thus, in the current study endotoxin (*E. coli* LPS) exposure did increase lung TLR4 mRNA expression and tended to increase lesions in the respiratory tract after IBV vaccination. Hence, chronic exposure to airborne LPS potentially affects disease susceptibility.

Unfortunately, in the design of the study we did not intend to sample nasal mucosa appropriately to analyze cytokine and TLR4 mRNA

expression. It would be interesting to know whether cytokine and TLR4 mRNA expression were altered in the nasal tissue as differences were found between HE and LE broilers for lesions in the nasal tissue. Thus, chronic exposure to high endotoxin (*E. coli* LPS) concentrations might sensitize the respiratory tract for damage upon exposure to a respiratory virus like IBV and increase disease susceptibility. Yet, lung lesions of Porcine Reproductive-Respiratory Syndrome Virus (PRRSV) infected pigs were little aggravated by subsequent LPS exposure (Van Gucht et al., 2003). Similarly, in the IBV vaccinated group no differences were found between HE and LE broilers for lesions in the trachea or lung. This might be related to the administration route used in the present study, in the first 19 days dust was used in combination with *E. coli* LPS, but from 19 days of age onwards *E. coli* LPS was sprayed without adding dust. A potential explanation might be that large particles are mainly found in the upper respiratory tract and smaller particles are distributed more homogeneously throughout the respiratory tract (Hayter and Besch, 1974). This might explain why we only see effects in the nasal tissue and not in the trachea or lungs. Furthermore, in the IBV challenged group no difference was found between HE and LE broilers for lesions in the nasal tissue, trachea or lungs. An explanation for this might be that the IBV challenge was more severe than the IBV vaccination (as indicated by ciliary activity and viral load), causing differences in lesions to be less pronounced between IBV challenged HE and LE broilers.

In the current study, chronic exposure to high endotoxin (*E. coli* LPS) concentrations did not clearly affect performance. Multiple studies in chickens, both laying hens and broilers, have shown that administration of LPS causes sickness symptoms including reduced body weight, body weight gain, feed intake, increased feed conversion ratio and more passive behavior (Cheng et al., 2004; Johnson et al., 1993; Xie et al., 2000). Yet, these sickness symptoms are often only reported within a relatively short time after administration of LPS (i.e. 3 days). Although we were not able to statistically analyze behavioral data, HE broilers seemed to show more passive behavior compared to LE broilers at 5 weeks of age and this was prior to IBV infection. Decreased activity could be an indication of pain (Gentle, 2011) and could further contribute to the development of lameness and leg pathologies, such as contact dermatitis (Bessei, 2006; Reiter and Bessei, 1998). Therefore, our finding could be an indication that chronic exposure to high endotoxin (*E. coli* LPS) concentrations might negatively affect animal welfare.

4.1. Endotoxin tolerance

Overall, broilers did not respond very strongly to chronic endotoxin (*E. coli* LPS) exposure with regard to the effects found on the immune system, respiratory tract and disease susceptibility. Repeated exposure to endotoxin has been shown to result in decreased immune responses in rodents and humans (Draisma et al., 2009; Freudenberg and Galanos, 1988; Kox et al., 2011; Zingarelli et al., 1995). This is known as endotoxin tolerance, i.e., the reduced capacity of an organism to respond to LPS after an initial exposure to this stimulus which occurs as a result of persistent TLR4 stimulation (Biswas and Lopez-Collazo, 2009). It results in a shift from pro-inflammatory to anti-inflammatory response, where TNFα, IL-12 and IL-6 expression is reduced and IL-10 and TGFβ expression is increased upon secondary stimulation (Biswas and Lopez-Collazo, 2009; Nahid et al., 2011). In birds, repeated systemic endotoxin exposure was shown to reduce the febrile response, IL-6 concentration and result in similar activity level as control birds (De Boever et al., 2008; Dudek et al., 2011; Marais et al., 2011). Furthermore, repeated exposure to LPS caused birds to become refractory to LPS (Korver et al., 1998; Lai et al., 2009; Parmentier et al., 2008, 2006). This might explain why chronic airborne endotoxin (*E. coli* LPS) exposure did not clearly affect performance in the present study. Yet, IL-10 mRNA expression was not altered by endotoxin (*E. coli* LPS) exposure and we did not investigate endotoxin effects on pro-inflammatory cytokines. Further research should identify whether levels of pro-inflammatory cytokines might be affected by chronic airborne endotoxin exposure, IBV treatment or their combination in chickens.

Current selective breeding programs focus mainly on breeding stronger and more productive broilers. The selection environments include commercial-like conditions to identify characteristics necessary to successfully adapt to challenging environments, including endotoxins. In the present study, *E. coli* LPS concentrations were continuously high, while in a commercial setting the endotoxin concentration would gradually increase. Broilers might be able to cope with the high endotoxin concentrations because they are selected in such an environment. Similarly, pigs seem to be able to cope with usual concentrations of LPS in pig houses without developing respiratory signs (Urbain et al., 1999). Furthermore, chicken lines have been shown to differ in their reaction to a LPS challenge (Cheng et al., 2004), indicating that selection could alter how chickens respond to LPS.

Overall, airborne chronic exposure to high endotoxin (*E. coli* LPS) concentrations seems to alter the immune system, specifically IFN-α and TLR4 mRNA expression in the lungs and systemic NAb binding PC-BSA. Previous studies suggested that LPS, either systemically or intratracheally administered, has immunomodulating features in broilers (Lai et al., 2009; Wideman et al., 2004). Thus, broilers could be more susceptible to infections, as seen by the increased lesions in the nasal tissue after infection with IBV in HE compared to LE broilers. In the present study we chose to use a virus infection instead of a bacterial infection as broilers were already continuously exposed to *E. coli* LPS. Furthermore, we wanted to test for disease susceptibility in the respiratory tract and not for bacterial infection in general. However, one could expect that broilers housed in the high endotoxin (*E. coli* LPS) environment and challenged with a bacterial infection could have worse disease symptoms, since TLR4 mRNA expression was altered. This was found in pigs that were intratracheally challenged with LPS and were inoculated with *Pasteurella multocida* type A 24 h later, where LPS predisposed pigs to a persistent lung inflammatory process and promoted *P. multocida* infection (Halloy et al., 2005). Thus, further research should focus on identifying effects of chronic airborne endotoxin exposure on subsequent bacterial infection.

5. Conclusion

In conclusion, chronic airborne exposure to high endotoxin (*E. coli* LPS) concentrations can affect components of the immune system and respiratory tract of broilers and could therefore influence disease susceptibility, and might further affect broiler welfare. Although broilers did not show any clear disease symptoms compared to previously found effects in humans, it should be considered to reduce endotoxin concentrations in animal husbandry systems as this would benefit both humans (farmers and local residents) and animals with regard to health and welfare.

Declaration of interest

None.

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