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Full Length Article

Effects of early nutrition and sanitary conditions on antibody levels in early and later life of broiler chickens

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ABSTRACT

Immune maturation of broiler chickens may be affected by management, such as early life feeding strategy (early versus delayed nutrition) or by low or high sanitary conditions (LSC versus HSC). We compared systemic maternal (MAb), natural (NAb), natural auto- (NAAb), and antigen specific antibody (SpAb) levels (IgM, IgY) between broilers (n = 48 per treatment) that received early (EN) or delayed nutrition for 72 h (DN) housed in either low (LSC) or high sanitary conditions (HSC) between 7 and 35 d of age. We found minimal interactions between feeding strategy and sanitary conditions. At 7 d of age, broilers receiving EN compared with DN, had elevated levels of IgM binding keyhole limpet hemocyanin (KLH), phosphoryl-conjugated ovalbumin (PC-OVA), and muramyl dipeptide (MDP), whereas effects of feeding strategy diminished at later ages. In LSC compared with HSC broilers, levels of NAb agglutinating RRBC and sheep red blood cells (SRBC) were already elevated from 14 d of age onwards. At 33 d of age, antibody levels (NAb, NAAb, anti-LPS, anti-MDP) were all elevated in LSC, compared with HSC broilers, for both IgM and IgY, but not IgM against KLH. Western blotting revealed different binding patterns of NAAb against chicken liver homogenate, which may indicate that the NAAb repertoire is affected by antigenic pressure. Our data suggest that antibody levels are affected for an important part by environmental conditions (feeding strategy and sanitary conditions), but minimally by their interaction. However, it remains to be further studied whether the enhanced levels of antibodies as initiated by EN and LSC contribute to enhanced resistance to infectious diseases.

1. Introduction

Activation and maturation of the immune system, including generation of antibodies, is suggested to be dependent on antigen exposure in the intestinal tract during the first days after hatch of broiler chickens (Bar-Shira et al., 2005; Bar-Shira and Friedman, 2006; Simon et al., 2014). Early exposure to intestinal microbiota and feed derived antigens may contribute to accelerated immune maturation (reviewed by Friedman et al., 2003). Therefore, the amount of these antigens after hatch may determine how fast antibody production will be established. Early (access to) nutrition (EN) and rapid exposure to dietary and microbial antigens in the intestinal tract as a result of feed intake (Binek et al., 2000; Karpinska et al., 2001; Potturi et al., 2005; Simon, 2016) may thus facilitate development of the immune system. In commercial broiler husbandry, however, broilers may experience a delay in access to feed and water up to 72h (DN), causing delayed maturation of the immune system (Bar-Shira et al., 2005), as antigen exposure is also in chickens essential for immune maturation (reviewed by Bar-Shira et al., 2003; Broom and Kogut, 2018). Studies comparing effects of EN versus DN on immune development, found accelerated maturation of the adaptive immune system, exemplified by higher lymphocyte numbers (Bar-Shira et al., 2005; Juul-Madsen et al., 2004), and earlier onset of antibody responses after rectal immunization (Bar-Shira et al., 2005). Whether EN contributes to improved later life immune responses, has been subject of debate. Most of these studies found little or no effects of EN on antibody levels (Dibner et al., 1998; Lamot et al., 2016; Simon et al., 2014; Walstra, 2011), but these studies were executed under relatively high sanitary conditions. Simon et al. (2015) observed lower antigen specific IgY responses in DN broilers, but not in EN broilers, housed under higher antigenic pressure compared with broilers housed under low antigenic pressure. This may indicate an interaction between feeding strategy and antigenic pressure on antibody responses in later life.

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Antibodies are important effector molecules of the immune system in both mammals and avian species (Vollmers and Brändlein, 2005), and are therefore good indicators for development and functioning of the immune system. Antibodies bind antigens and activate the complement cascade, resulting in neutralization of pathogens and removal of immune complexes by phagocytic cells (Ochsenbein and Zinkernagel, 2000). Three types of antibodies are distinguished: specific antibodies (SpAb) after antigenic stimulation, natural antibodies (NAb), and natural auto- (or self-binding) antibodies (NAAb). Classical SpAb responses rest on T-cell help after antigen presentation by antigen presenting cells, resulting in SpAb secreting plasma cells, with increasing affinity and specificity, and memory B-cells (Tizard, 2018). The existence of NAb, which bind to antigens to which the immune system has never been exposed, has been demonstrated in chicken (Matson et al., 2005; Parmentier et al., 2004). Natural antibodies have been found to contribute to resistance against bacterial pathogens, and higher levels of NAb correlated with reduced risk of mortality in laying hens (Berghof et al., 2019; Star et al., 2007; Sun et al., 2011). Antibodies binding towards (altered) self-antigens (NAAb) are present in chickens as well (Bao et al., 2016; De Jong et al., 2014; van der Eijk et al., 2019; Van Dijk and Parmentier, 2020), although their exact function in chickens need to be unraveled.

It is unknown whether early exposure of the immune system to a greater antigenic load may affect levels of NAb and NAAb, and whether differences persist on the long term under low (LSC) or high sanitary conditions (HSC). To our knowledge, studies that compare immune development of broiler chickens under either LSC or HSC, and the interaction with feeding strategy are rare (Simon et al., 2015). Hence, we studied whether different feeding strategies (EN versus DN) and sanitary conditions (LSC versus HSC) affect development of NAb, NAAb, and SpAb levels. We propose that EN compared with DN, due to increased antigenic exposure, led to earlier stimulation of antibody producing B-cells, and thus higher antibody levels in EN broilers. This may ultimately improve the first line of defense towards infections and survival (Berghof et al., 2019; Star et al., 2007; Sun et al., 2011), and enhanced physiological homeostasis due to removal of damaged auto-antigens (Lutz et al., 2009; Ochsenbein, 1999).

2. Materials and methods

2.1. Experimental design

The experiment and procedures were ethically approved according to Dutch law under application number AVD104002016441. The experiment was executed in 3 consecutive batches and designed as a 2*2 factorial approach consisting of feeding strategy (EN versus DN) and sanitary conditions (LSC versus HSC). One separate climate respiration chamber (CRC) was used for each sanitary condition and each CRC contained 8 floor pens (1.1 * 1.8 m), each containing 10 broilers per pen at the start of the experiment (0 d of age). This resulted in a total of 16 pens divided over 2 CRC. Both CRC were completely identical in their set-up and were controlled for identical climate conditions (temperature, humidity, CO_2, NH_3). During the first 7 d of age, 1 broiler per pen was randomly selected and euthanized for another study (*Hollemans* et al., *manuscript in preparation*) at 0, 1, 2, 3, and 7 d of age. The broiler euthanized at 7 d of age, was used for blood plasma and liver collection for antibody measurements and Western blotting. From 7 d of age onwards, pens contained 5 broilers per pen. At 24 d of age, 1 broiler per pen was randomly selected for sheep red blood cell (SRBC) immunization and blood serum collection. At 33 d of age, 1 broiler per pen was randomly selected out of the remaining 4 broilers in the pen for blood plasma and liver collection. Random selection of broilers was done with the random sampling procedure in R version 3.6.1. (R Development Core Team, 2018).

2.2. Animals, housing, and nutrition

For each respective batch, just hatched (<4 h) Ross 308 male hatchlings (n = 160/batch) without abnormalities (parent stock age: batch 1: 31 w, batch 2: 33, and batch 3: 48 weeks) were obtained from a commercial hatchery, and transported to the experimental facility. After arrival, chickens received an ID tag in the neck, and were distributed over floor pens. The pens contained SoftCell (Agromed GmbH, Kremsmünster, Austria) as bedding material, covered with chicken paper during the first 3 d to prevent litter uptake. Ambient temperature was set at 36 °C and was gradually reduced to 29 °C until 7 d of age, and then further gradually reduced to 18 °C at 42 d. Relative humidity was set at 55% at the start of the trial and gradually increased to 75% at 42 d. Levels of CO_2 were maintained $\leq$2500 ppm and that of $NH_3 \leq 20$ ppm. Broilers had *ad libitum* access to water via drinking nipples and feed via a feeder, except for DN chickens, which had no access to nutrition (water and feed) during the first 72 h after hatch from placement onwards. Commercial pelletized broiler starter (0–7 d; DE: 2850 kcal/kg; total lysine: 11.8 g/kg), grower (7–28 d; DE: 2900 kcal/kg; total lysine: 11.2 g/kg), and a finisher diets (28–35 d; DE: 2950 kcal/kg; total lysine: 10.7 g/kg) were fed. The grower diet contained decoquinate (0.05 g/kg; Deccox 6%, Zoetis, Capelle aan den IJssel, the Netherlands). Broilers were vaccinated against Newcastle disease at 3 d of age, but this was accidently omitted in batch 1. We observed no indications that this may have influenced the outcome of the experiment.

2.3. Induction of low and high sanitary conditions

Induction of different sanitary conditions is described in detail elsewhere (Hollemans et al., *in preparation*). In short, LSC were induced from 3 d of age until the end of the experiment and consisted of spreading used litter (from 3 commercial broiler flocks per batch, obtained at approximately 35 d) in pens every 4 d, and the CRC was under-pressurized (- 65 $\pm$ 5 Pa). Broilers housed under HSC were kept in a separate over-pressurized (100 $\pm$ 5 Pa) CRC and caretakers and researchers were obliged to shower, and wear clean clothes, hairnet, gloves, and disinfected boots.

2.4. Immunizations

To induce a classical specific immune response, 2 broilers per pen received an intra-muscular (i.m.) immunization with 1 mL of 25% packed sheep red blood cells (SRBC) in phosphate buffered saline (PBS) at 24 d of age (0 d post immunization (p.i.)). Packed SRBC were obtained by washing SRBC 5 times with PBS. Centrifugation was done at 1000 g for 15 min. Final packed SRBC were diluted in sterile PBS and stored at 4 °C upon immunization (within 24 h).

2.5. Sample collection

Whole blood from 1 broiler per pen was collected at 14, 24, and 31 d of age (−10, 0, 7 d p.i.), incubated for 2 h at 4 °C, and subsequently centrifuged (12,000 g, 5 min) to obtain serum for haemagglutination assays. In batch 2 and 3, whole blood from 1 broiler per pen was collected in heparinized tubes, and centrifuged (12,000 g, 5 min) to obtain plasma at 7 and 33 d of age, for ELISA and Western blotting. Afterwards, the broiler was euthanized by decapitation (7 d) or intravenous injection with an overdose of pentobarbital (33 d), and approximately 5 g of liver was collected in cryovials, and snap frozen in liquid nitrogen. All plasma and serum samples were stored at −20 °C, and liver samples were stored at −80 °C, until further analyses.

2.6. Enzyme-linked immunosorbent assay

Flat-bottomed 96-well medium binding ELISA plates (Greiner Microlon, Sigma-Aldrich, Darmstadt, Germany) were coated with 100

μL/well of antigen dilutions in coating buffer (pH 9.6) and incubated at 4 °C overnight. Antigens were either PC-OVA (2 μg/mL, Santa Cruz Biotechnology, SC-396491), KLH (2 μg/mL, Sigma-Aldrich, H7017), LPS (2 μg/mL, Sigma-Aldrich, L2880), and MDP (1 μg/mL, Sigma-Aldrich, A9519). After washing with PBS, plates were filled with 100 μL of PBS containing Tween 20 (0.05%) and horse serum (1%) per well. Plasma (starting at 1:40 dilutions) was added followed by 4 dilution steps, as well as a standard positive control (*in duplo*) from pooled plasma, and plates were incubated for 1.5 h at 20 °C and subsequently washed with tap water. Conjugates (goat-*anti*-chicken IgM or goat-*anti*-chicken-IgY conjugated to horse radish peroxidase (Bethyl Laboratories Inc., Montgomery, TX); all 1:10,000 diluted for all antigens, and 1:40,000 for LPS-IgY) were added to the plates and incubated for 1.5 h at room temperature. After washing, binding of antibodies was visualized by adding 100 μl of substrate (reverse osmosis purified water, 10% tetramethylbenzidine buffer (15.0 g/L sodium acetate, 1.43 g/L urea hydrogen peroxide; pH 5.5), and 1% tetramethylbenzidine (8 g/L DMSO) at 20 °C. After 15 min, the reaction was terminated with 50 μl of 1.25 M H_2SO_4. Extinctions were measured with a Multiskan GO (Thermo scientific, Breda, the Netherlands) at 450 nm. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of the standard positive.

2.7. Haemagglutination assay

Haemagglutination and lysis of SRBC and rabbit red blood cells (RRBC) were analyzed in serum collected at 14, 24, and 33 d of age following procedures of Matson et al. (2005), including treatment of serum with β-mercaptoethanol (2-ME) to cleave 2-ME sensitive antibodies for 30 min at 37 °C. Normal and 2-ME treated sera were then diluted with PBS in a twofold serial dilution in 96-well round bottom assay plates, resulting in a total of 11 dilutions, including PBS as a negative control, in a reaction volume of 25 μL. To all wells, 25 μL of 1% RRBC (Innovative Research, Novi, U.S.A., IRBRBC25ML) or SRBC (ThermoFisher Scientific, Bleiswijk, the Netherlands, S0051D) suspension was added. Then the plates were gently vortexed for 10 s, and incubated for 24 h at room temperature. After incubation, the highest dilution showing haemagglutination, was scored. The ratio of levels of antibodies binding SRBC between 7 and 0 d post SRBC immunization was calculated to obtain fold change.

2.8. SDS-PAGE and western blotting

Preparation of chicken liver homogenate (CLH) was done following procedures of Van Dijk and Parmentier (2020). In short, 0.5 g of liver tissue was homogenized in 5 mL buffer (50 mM Tris, 100 mM NaCL, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 2% glycerol, pH 7.4) and 2.5 mL of protease inhibitor cocktail (Sigma-Aldrich). After the mixture was centrifuged (13,500 g, 15 min), supernatants were diluted 1:30 with PBS, incubated (95 °C, 5 min) with β-mercaptoethanol and Laemmli buffer (BIORAD, Hercules, CA, USA), and stored at −20 °C until further use.

Binding of IgM and IgY antibodies to CLH antigens in plasma of birds at 33 d of age by Western blotting after protein separation SDS-PAGE on 4–15% precast gels (BIORAD), was done following procedures as previously described (Van Dijk and Parmentier, 2020). A negative control was added to all gels by means of replacing plasma by PBS. Molecular weights were estimated by a color standard (10–250 kD, BIORAD). After scanning the blots with a flatbed scanner (GS-600, BIORAD), stained CLH fragments (bands) were counted by Image Lab 6.0 software (BIORAD). To know whether feeding and sanitary condition treatments affected the antigen composition of CLH contents, binding of antibodies was tested on CLH obtained from all treatments (EN-LSC, EN-HSC, DN-LSC, DN-HSC, n = 4 randomly selected broilers per treatment). As there were no effects of the experimental treatments on CLH fragment composition (Supplementary Data), CLH from one adult laying hen (16 w of age) was used in all Western blot assays.

2.9. Statistical analyses

Data were processed, analyzed, and presented using R version 3.6.1 (R Development Core Team, 2018). General linear models were established to estimate fixed effects of sanitary conditions (LSC, HSC), feeding (DN, EN), and their interaction, on levels of antibodies binding KLH, PC-OVA, LPS, and MDP for 7 and 33 d of age separately. Identical models were established to analyze treatment effects on performance data (BW, ADG) on agglutination of SRBC and RRBC including fold change, and number of bands detected on Western blots. In all models, batch was added as a covariate including a batch * feeding and a batch * sanitary conditions interaction. Non-significant covariates ($P \leq 0.10$) were excluded from the model.

Model residuals of the linear models were tested to verify assumptions of normality and homogeneity by QQ-plots and residual plots. Logarithmic transformation was applied to normalize residuals if required. P-values ≤ 0.05 were considered statistically significant and P-values ≤ 0.10 were considered as tendencies. All data are presented as (back-transformed) estimated marginal means with standard errors unless specified otherwise.

3. Results

3.1. Antibody levels

At 7 d of age we measured plasma levels of IgM and IgY binding KLH, RRBC, and SRBC before immunization (as a parameter for NAb), PC-OVA (NAAb), LPS and MDP (Table 1, Figs. 1 and 2), to study effects of early life feeding strategy (EN versus DN) on early life antibody levels and whether or not these are affected by sanitary conditions (LSC versus HSC). At 33 d of age, we again measured levels of antibodies (IgM, IgY) binding KLH, PC-OVA, LPS, and MDP (Table 2). Here, we investigated whether differences caused by feeding strategy at 7 d of age lasted up to 33 d of age, and whether sanitary conditions during rearing affect antibody levels at later life. Haemagglutination against SRBC after immunization (SpAb) was measured in serum to compare the classical specific antibody response between all treatment groups (Fig. 1). With regard to all antigens and ages, no interaction effects (feeding strategy * sanitary conditions) were present ($P > 0.10$), with exception of NAb binding RRBC at 24 d of age ($P = 0.08$), and therefore results will be presented separately for feeding strategy and sanitary conditions.

3.1.1. NAb

At 7 d of age, levels of natural IgM, but not IgY, binding KLH tended ($P = 0.08$) to be higher in EN compared with DN broilers ($\Delta = 0.6$; Table 1), whereas at 33 d of age no effects were observed (Table 2). Levels of IgY, but not IgM, binding KLH were lower in LSC compared with HSC at 7 d ($\Delta = 0.8$; $P = 0.09$), but at 33 d of age levels of LSC were higher compared with HSC ($\Delta = 2.4$; $P < 0.001$). Agglutination of RRBC was measured at 14, 24, and 31 d of age to study levels of NAb binding RRBC during aging, and were not affected by feeding strategy at any age (Fig. 2). In LSC compared with HSC broilers, levels of RRBC agglutination tended to be higher ($P = 0.10$) at 24 d of age, and were higher ($P = 0.01$) at 31 d of age. Levels of NAb binding SRBC at 14 d of age were higher in both untreated ($P < 0.01$) and 2-ME treated serum ($P = 0.07$).

3.1.2. NAAb

At 7 d of age, IgM binding the auto-antigen PC-OVA was higher ($\Delta = 0.9$; $P = 0.01$) in EN compared with DN, while IgY was lower ($\Delta = 1.4$; $P = 0.005$). No effects of sanitary conditions were found. At 33 d of age, LSC compared with HSC broilers had higher levels of both IgM ($\Delta = 2.4$, $P < 0.001$), and IgY ($\Delta = 1.6$, $P = 0.01$) binding PC-OVA, whereas no effects of feeding strategy were found.

Table 1
IgM and IgY titers measured by ELISA binding the antigens KLH, PC-OVA, LPS, or MDP in plasma of 7 d old broiler chickens receiving either delayed (DN) or early nutrition (EN), kept under high (HSC) or low sanitary conditions (LSC). Data are presented as estimated marginal means with standard errors (SEM). KLH = keyhole limpet hemocyanin, PC-OVA = phosphoryl choline – ovalbumin, LPS = lipopolysaccharide from E. coli, MDP = muramyl di-peptide.

Antigen	Isotype	Treatments											Fixed effects[a]				
		Delayed nutrition HSC			Delayed nutrition LSC			Early nutrition HSC			Early nutrition LSC						
		Mean	SEM	n[b]	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	SC * Feeding	Feeding	SC	Batch * SC	Batch
KLH	IgM	0.2	0.3	8	0.2	0.3	8	0.7	0.3	8	0.9	0.3	8	0.73	0.08	0.70	0.07	0.01
	IgY	6.0	0.5	8	4.9	0.4	8	5.1	0.4	8	4.6	0.4	8	0.54	0.21	0.09	0.05	0.55
PC-OVA	IgM	1.3	0.3	8	1.7	0.3	8	2.4	0.3	8	2.3	0.3	8	0.38	0.01	0.54	-[c]	0.94
	IgY	10.7	0.4	8	10.5	0.4	8	9.3	0.4	8	9.1	0.4	8	0.92	< 0.01	0.59	–	0.07
LPS	IgM	0.9	0.6	8	1.0	0.6	8	1.2	0.6	8	1.3	0.6	8	0.99	0.62	0.82	–	0.87
	IgY	5.0	0.4	8	4.2	0.4	8	4.4	0.4	8	4.1	0.4	8	0.51	0.35	0.15	–	0.09
MDP	IgM	0.7	0.3	7	0.8	0.3	7	2.1	0.3	8	1.7	0.3	8	0.41	0.01	0.61	–	0.31
	IgY	6.3	0.3	7	5.7	0.3	7	5.6	0.3	7	5.6	0.3	8	0.35	0.14	0.37	–	0.04

[a] Model established P-values for fixed effects of feeding, sanitary condition, batch, and their two-way interactions.
[b] Number of replicate broilers, housed with 5 broilers per pen.
[c] In the case of P-values > 0,10 for batch * SC interactions, the fixed effect was left out of the model.

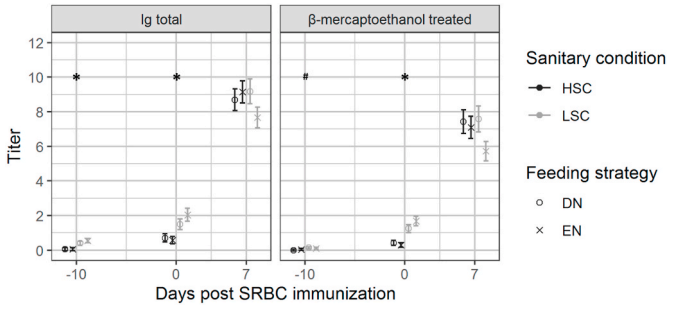

Fig. 1. Agglutination of sheep red blood cells (SRBC) by untreated (Ig Total) or β-mercaptoethanol treated serum from broiler chickens receiving delayed (DN) or early nutrition (EN) and kept under high (HSC) or low sanitary conditions (LSC), at -10, 0, and 7 d post i.m. immunization with SRBC. Timepoints with * indicate significant (P ≤ 0,05) effects of sanitary conditions, while # indicates tendencies (P ≤ 0,10) for differences between sanitary conditions. n = 12 replicate broiler housed in groups of 5 broilers per pen for all groups, except for 7 and 11 d post immunization, where n = 10 replicate broilers for all groups with exception of DN-HSC (n = 12 broilers).

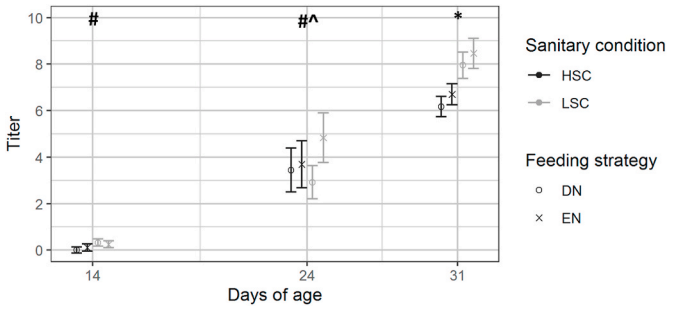

Fig. 2. Agglutination of rabbit red blood cells (RRBC) by untreated serum from broiler chickens receiving delayed (DN) or early nutrition (EN) and kept under high (HSC) or low sanitary conditions (LSC), at 14, 24, and 31 d of age. Broilers were i.m. immunized with sheep red blood cells (SRBC) at 24 d of age (0 d post immunization). Timepoints with * indicate significant (P ≤ 0,05) effects of sanitary conditions, while # indicates tendencies (P ≤ 0,10) for differences between sanitary conditions, and #^ indicates tendencies for interactions between sanitary condition and feeding strategy. n = 12 replicate broiler housed in groups of 5 broilers per pen for all groups, except for 7 and 11 d post immunization where n = 10 replicate broilers for all groups with exception of DN-HSC (n = 12 broilers).

3.1.3. *Antibodies binding LPS and MDP*

Antibodies binding LPS or MDP are difficult to define as either NAb or SpAb, because the broilers are expected to be exposed to LPS and MDP. It is however uncertain whether this is the case for the antigens used in the ELISA. Therefore, no distinction has been made between NAb and SpAb for these antigens. At 7 d of age, we observed that IgM and IgY antibodies binding LPS from *E. coli* were not affected by feeding strategy or sanitary conditions. Levels of IgM binding MDP were higher (Δ = 1.1; P = 0.01) in EN compared with DN broilers, whereas IgY binding MDP was unaffected by early life feeding strategy. Both IgM and IgY binding MDP were not affected by sanitary conditions. At 33 d of age, levels of IgM binding LPS tended to be lower (Δ = 0.7; P = 0.07) in EN compared with DN, but IgY binding LPS and antibodies binding MDP were unaffected by feeding strategy. In 33 d old broilers, we observed higher levels of IgM binding LPS from *E. coli* (Δ = 1.0; P = 0.01) and IgY (Δ = 3.1; P = 0.002) in LSC compared with HSC broilers. Antibodies binding MDP were higher (P < 0.001) in LSC compared with HSC broilers, for both IgM (Δ = 1.2) and IgY (Δ = 1.5).

3.1.4. *Specific antibody levels*

Agglutination of SRBC before (-10 and 0 d p.i., as measure of NAb) and after (7 d p.i., as a measure of SpAb) immunization with SRBC in untreated and 2-ME treated serum (Fig. 1) was not affected by feeding strategy (EN versus DN), while we observed higher levels of agglutination in LSC compared with HSC broilers in both untreated and 2-ME treated serum before immunization (NAb), but not at 7 d p.i. (SpAb). Because of different basal levels of agglutinating antibodies prior to immunization, the fold change between 0 and 7 d p.i. Was calculated. We observed higher fold change in untreated (Δ = 2.0; P = 0.02) serum of HSC compared with LSC, and 2-ME treated serum (Δ = 2.0; P < 0.001) in HSC compared with LSC. We found a tendency (P = 0.06) for an interaction (feeding strategy * sanitary conditions) that indicated the lowest fold change in 2-ME treated serum of EN-LSC compared with other groups. Sheep red blood cell lysis titers varied between 0 to 2 among all ages, and were not different between treatments (data not shown).

3.2. *Antibodies binding chicken liver lysate*

Western blots were conducted to study whether feeding strategy and sanitary conditions affected antibody binding patterns to a pool of liver antigen fragments at 33 d of age, thereby reflecting the NAAb repertoire (Vaz, 2000). We observed divergent IgM and IgY (Fig. 3) patterns binding CLH, which was confirmed by higher number of IgM and IgY

Table 2
IgM and IgY titers measured by ELISA binding the antigens KLH, PC-OVA, LPS, or MDP in plasma of 33 d old broiler chickens receiving either delayed (DN) or early nutrition (EN), kept under high (HSC) or low sanitary conditions (LSC). Data are presented as estimated marginal means with standard errors (SEM). KLH = keyhole limpet hemocyanin, PC-OVA = phosphoryl choline – ovalbumin, LPS = lipopolysaccharide from E. coli, MDP = muramyl di-peptide.

Antigen	Isotype	Treatments											Fixed effects[a]			
		Delayed nutrition HSC			Delayed nutrition LSC			Early nutrition HSC			Early nutrition LSC					
		Mean	SEM	n[b]	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	SC * Feeding	Feeding	SC	Batch[c]
KLH	IgM	3.4	0.4	6	4.1	0.4	7	3.6	0.4	8	3.4	0.4	8	0.25	0.70	0.55	0.75
	IgY	2.1	0.3	8	4.4	1.0	7	1.8	0.3	8	4.3	0.7	8	0.69	0.79	< 0.001	0.69
PC-OVA	IgM	7.4	0.5	8	9.6	0.7	7	6.8	0.5	8	9.5	0.6	8	0.57	0.53	< 0.001	0.01
	IgY	6.9	0.5	8	8.9	0.5	7	7.3	0.5	8	8.6	0.5	8	0.50	0.89	0.01	0.07
LPS	IgM	6.5	0.3	8	7.5	0.4	6	5.8	0.3	8	6.8	0.3	7	0.90	0.07	< 0.001	0.80
	IgY	2.3	0.7	8	6.1	0.7	6	2.3	0.7	8	4.6	0.7	7	0.58	0.62	< 0.01	0.09
MDP	IgM	5.3	0.3	8	6.7	0.4	6	5.7	0.3	8	6.8	0.4	7	0.63	0.48	0.001	< 0.001
	IgY	3.4	0.3	4	5.1	0.4	2	3.8	0.3	4	5.2	0.3	3	0.60	0.49	< 0.001	0.04

[a] Model established P-values for fixed effects of feeding, sanitary condition, batch, and their two-way interactions.
[b] Number of replicate broilers, housed with 5 broilers per pen.
[c] There were no interaction with batch.

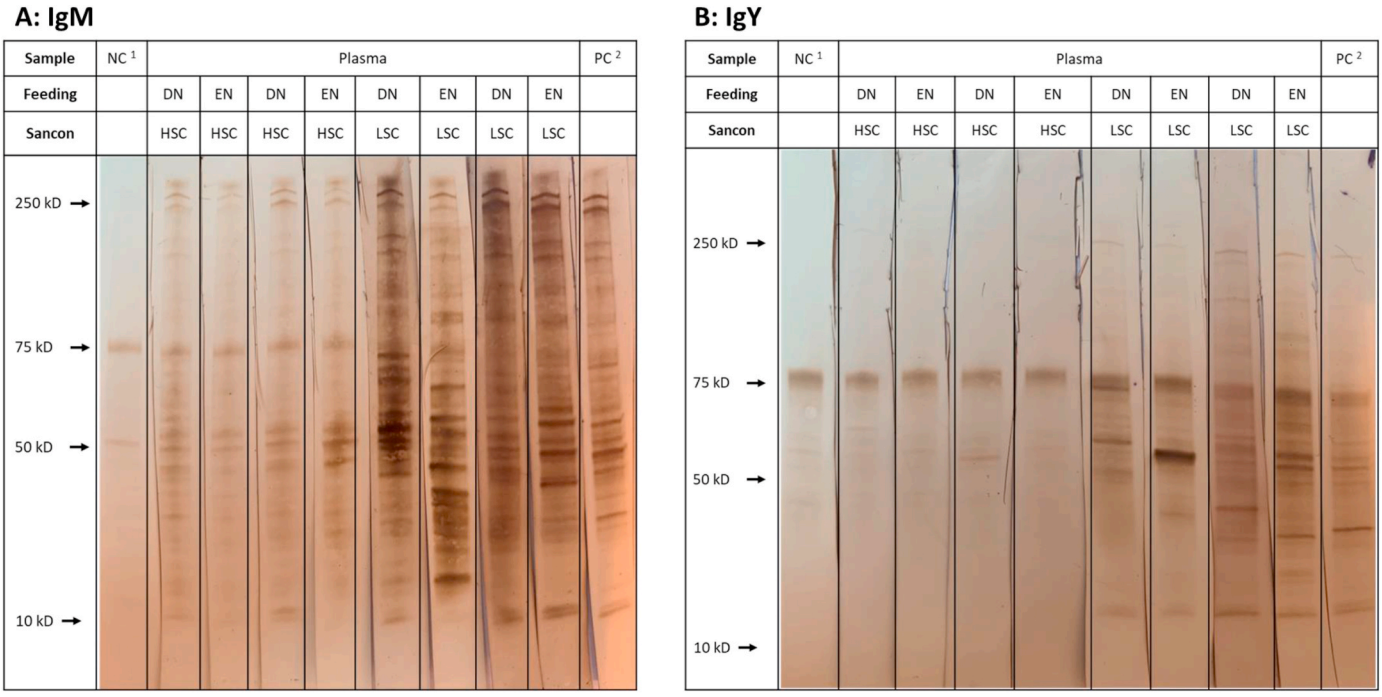

Fig. 3. Western blots representing immunoglobulin M (A) or immunoglobulin Y (B) binding chicken liver homogenate (CLH) in plasma collected at 33 d of age in broiler chickens receiving delayed (DN) or early nutrition (EN) and kept under high (HSC) or low sanitary conditions (LSC). Each lane represents CLH binding profile of an individual broiler. Western blots are representative for all 4 broilers measured per treatment.

1 NC = Negative control (phosphate buffered saline instead of plasma).
2 PC = Positive control containing reference plasma from an adult layer hen (16 w of age).

stained bands by LSC compared with HSC broilers (both $P < 0.001$; Table 3). For both isotypes, we did not find differences between EN and DN, but differences between sanitary conditions (LSC versus HSC) were clearly present. Western blots revealed more liver fragments stained and higher staining intensity of these fragments by IgM and IgY from birds kept under LSC, compared with HSC, indicating that higher levels of self-binding antibodies are present under LSC.

4. Discussion

We studied effects of feeding strategy (EN versus DN) and sanitary conditions (LSC versus HSC) on the presence and development of maternal (mAb), natural (NAb), natural-auto (NAAb), and specific antibody (SpAb) levels in broiler chickens. Specifically, we measured levels of antibodies (IgM, IgY) binding (1) KLH, RRBC, and SRBC before immunization, as parameters for natural antibodies (NAb), (2) PC-OVA as parameter for natural auto-antibodies (NAAb), and (3) LPS and MDP as an indication of exposure to microbial associated molecular patterns. In addition, we measured specific antibodies (SpAb) binding SRBC after immunization with SRBC, to study adaptive antibody responses, and Western blotting was performed to study the NAAb repertoire binding CLH. Interactions among feeding strategy and sanitary conditions were

Table 3
Number of stained bands of immunoglobulins M (IgM) and Y (IgY) in Western blots, binding chicken liver homogenate in 33 d old broiler chickens housed under low (LSC) or high sanitary conditions (HSC). Data are presented as raw means with standard deviation (SD).

Isotype	LSC		HSC		Sanitary condition[a]
	Mean	SD[1]	Mean	SD	
IgM	27.3	3.0	20.8	5.1	<0.001
IgY	18.4	4.9	7.1	1.6	<0.001

[a] Model established P-value, there were no significant batch effects.n = 16 replicate broilers per group, divided over 2 batches.

not present for any of the parameters, indicating that the observed short-term effects of EN, do not affect antibody levels when housed under LSC conditions. In EN, compared with DN broilers, we observed increased IgM levels of NAb and NAAb, and antibodies binding MDP at 7 d of age. These differences were no longer present at 33 d of age suggesting no long-term effects of EN on the humoral immune system. Broilers reared under LSC compared with HSC, had higher levels of NAb agglutinating SRBC (-10 and 0 d p.i.) and RRBC (14, 24 and 31 d of age), as well as higher levels of NAb binding KLH, NAAb binding PC-OVA, and antibodies binding LPS and MDP. Absolute levels of SpAb agglutinating SRBC were not affected by treatments, but a higher fold change of SpAb agglutinating SRBC was observed in HSC, compared with LSC broilers. Effects of treatments on growth performance have been published before (Hollemans et al., 2020).

4.1. Maternal IgY

In chickens, maternal antibodies (mAb) are transferred via both yolk (IgY) and albumen (IgM and IgA) (Hamal et al., 2006; Ismiraj et al., 2019; Van Dijk and Parmentier, 2020). Studies investigating yolk utilization after hatch, suggested better utilization of yolk constituents after EN compared with DN broilers (Noy et al., 1996; Noy and Sklan, 2001), potentially increasing the uptake of mAb from yolk in the first days after hatch. However in this study we found no differences in residual yolk disappearance among EN and DN broilers at 0, 1, 2, 3, and 7 d of age (data not shown). It appears that no consensus has been reached yet on effects of EN on yolk disappearance, and the reasons for the inconsistent findings among studies are yet unknown (reviewed by van der Wagt et al., 2020). So far, it is unknown whether maternal IgY deriving from the yolk is transported through the intestinal epithelium. To obtain a first insight whether uptake of mAb still might be affected by EN, we tested whether EN compared with DN broilers, have higher levels of mAb in blood plasma, which may contribute to passive (maternal) protection. We assume that the measured IgY at d 7 is of maternal origin, as IgY secreting neonatal B-cells are not detectable in spleen up to 6 d of age (Lawrence et al., 1981), and IgY gene expression was not detectable in intestinal tissue up to 10 d of age (Lammers et al., 2010). Levels of systemic IgY binding KLH, LPS, and MDP were not affected by feeding strategy, providing a first suggestion that mAb uptake is not enhanced after EN. Speculatively, our observation of lower levels of IgY binding PC-OVA in EN compared with DN broilers, might suggest greater turnover of NAAb in EN broilers but this cannot be confirmed within this study.

4.2. Specific antibody responses

Whereas levels of SpAb binding SRBC were unaffected by sanitary conditions at 31 d of age, fold change ratio in levels of SpAb binding SRBC between 0 and 7 d p.i. (24 and 31 d of age) was lower in LSC compared with HSC broilers. As levels of NAb binding SRBC were higher in LSC compared with HSC broilers at 24 d of age (0 d p.i.), we suggest that in LSC broilers, NAb levels are elevated, while HSC broilers have lower NAb levels and generate higher SpAb responses. This observation was also reported in repeatedly immunized laying hens, (Parmentier et al., 2002). Also in goldfish with high levels of NAb, compared with goldfish having low levels of NAb, had lower fold change of antibodies after immunization (Sinyakov et al., 2002). These observations may imply that dependent on the sanitary conditions, different immune strategies are used. Under LSC, pre-existing antibodies (NAb) act as a first line of defense, whereas under HSC stronger adaptive antibody responses are required. Main effects of feeding strategy on SpAb responses were absent, and therefore in line with previous research (Lamot et al., 2016; Simon et al., 2015).

4.3. Natural (auto) antibodies

In the current study, we observed that both feeding strategy (EN versus DN) and sanitary conditions (LSC versus HSC), affected levels of systemic antibodies (NAb, NAAb, anti-LPS and anti-MDP). Effects of feeding strategy, however, were only present at 7 d of age, whereas effects of sanitary conditions were present from 14 d of age until the end of the experiment (33 d of age). We observed no interactions between feeding strategy and sanitary conditions, suggesting that EN broilers do not respond differently to LSC than DN broilers.

4.3.1. Early versus delayed nutrition

As IgM is barely transferred maternally (Ismiraj et al., 2019), the detected IgM at 7 d is likely endogenously produced. Immunoglobulin M secreting B cells were demonstrated to be present from 3 d of age onwards (Lawrence et al., 1981). The observed higher levels of IgM binding KLH, PC-OVA, and MDP in EN compared with DN broilers at 7 d of age, indicate accelerated maturation of the neonatal humoral immune system during the early life. Greater levels of NAb (anti-KLH, anti-MDP) suggest that EN compared with DN, improves defense towards infections at young ages (Berghof et al., 2019) and reduces risk of mortality (Star et al., 2007; Sun et al., 2011), at least up to 7 d of age. Whether the increased levels of NAAb (anti-PC-OVA) after EN at 7 d of age may contribute to better regulation of immune responses towards auto-antigens, remains unclear. Natural auto-antibodies are expected being responsible for clearance of apoptotic or senescent cells and damaged auto-molecules, preventing auto-immune responses and chronic inflammation in humans (Lutz, 2007; Nagele et al., 2013; Xu et al., 2015). The implications for chickens are, however, yet unclear. Simon et al. (2014) found no effects of feeding strategy up to 9 d of age on levels of NAb, but observed lower levels of natural IgM binding KLH in EN, compared with DN broilers, at 14 d of age. So far we have no explanation for this inconsistency. From 14 d onwards, we observed no effects of EN, compared with DN, on NAb binding SRBC or RRBC, which is in accordance with most other studies on NAb binding SRBC (Lamot et al., 2016) or KLH (Lamot et al., 2016; Simon et al., 2015, 2014), that reported no long-term (21 to 28 d of age) effects of feeding strategy on NAb levels.

Our observation that EN compared with DN enhances levels of systemic antibodies (NAb, NAAb, anti-LPS and anti-MDP) may be caused by higher numbers of T and B-cells in the bursa after EN, compared with DN (Bar-Shira et al., 2005). These authors suggested the higher T end B cell numbers to be the result of increased exposure to antigens (derived from ingested feed and bacterial colonization) in EN. The findings of Bar-Shira et al. are supported by another study (Haghighi et al., 2006) that reported higher NAb levels in 14 d old broilers receiving probiotic bacteria immediately after hatch. In mammals, activation of Toll-like receptors (TLR) on B-cells contributes to differentiation of naïve B-cells to antibody-secreting plasma cells (Bekeredjian-Ding and Jego, 2009; Bernasconi et al., 2003; Kreuk et al., 2019; Ruprecht and Lanzavecchia, 2006), which is also suggested to be the case in chickens (St. Paul et al., 2012). Thus, greater exposure to antigens and TLR ligands in EN compared with DN broilers, may cause greater stimulation of naïve B-cells, and differentiation into plasma cells, eventually resulting in higher levels of systemic antibodies. Altogether, our data confirms that

EN enhances maturation of the humoral immune system, but only temporarily (up to 7 d of age).

4.3.2. Low versus high sanitary conditions

At 7 d of age, we observed no effects (all $P > 0.10$) of sanitary conditions on antibody levels. The contrast in sanitary conditions was applied from 3 d of age onwards, which may have been too short to cause differences at 7 d of age. Levels of NAb agglutinating SRBC and RRBC were higher in LSC compared with HSC from 14 d of age onwards (Figs. 1 and 2). This was also reflected at 33 d of age by higher ($P < 0.001$) levels of IgY binding KLH in LSC compared with HSC broilers. Furthermore, levels of both IgM and IgY binding PC-OVA (NAAb), LPS, and MDP were higher (all $P < 0.01$) in LSC groups. Higher antigenic pressure in LSC compared with HSC broilers, likely resulted in greater activation of B-cells. In addition, by Western blotting we revealed different binding patterns of both IgM and IgY against CLH between LSC and HSC broilers. These observations indicate that differences in antigenic pressure during rearing (LSC versus HSC), might affect the binding repertoire against auto-antigens. Especially the binding pattern of IgY differed between sanitary conditions, showing a greater range of bound liver antigens in LSC compared with HSC broilers (Table 3). Our data suggest that in chickens, environmental components (such as antigenic pressure) alter antibody levels (NAb and NAAb) and repertoire (NAAb), apart from the genotype (de Jong et al., 2013; Parmentier et al., 2017). The increased levels of NAAb under LSC, could represent an adaptive mechanism to maintain immune homeostasis under high antigenic pressure. However, the exact mechanism regulating NAAb levels in LSC, compared with HSC broilers, remains unclear and requires further study, as the origin and exact function of NAAb in chickens remains unknown.

5. Conclusions

We demonstrated marginal interaction effects of feeding strategy (EN versus DN) and sanitary conditions (LSC versus HSC) on antibody levels (SpAb, NAb, NAAb) up to 35 d of age in broiler chickens. Early nutrition, compared with DN, enhanced levels of NAb and NAAb up to 7 d of age, while effects of LSC compared with HSC were observed from 14 d of age onwards. Our study suggests that antibody levels (SpAb, NAb, NAAb, anti-LPS and anti-MDP) are affected for an important part by environmental conditions (feeding strategy and sanitary conditions). It remains to be studied which components of EN and LSC affect humoral immunity, although greater exposure to antigens is the most likely route.

Declaration of competing interest

MH is employee of Coppens Diervoeding B·V.. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103954.

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