

# **Effects of early life conditions on immunity in broilers and layers**



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# **Effects of early life conditions on immunity in broilers and layers**

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## ABSTRACT

The course for later life immune responses is set early in life during the developmental phase of the immune system and accordingly disturbances of immune development may have long-term consequences for host health. In terms of immune activation and immune development the gut microbiota play an important role and consequently disturbances of early life microbial colonization may affect host immunity later in life. In chickens, disturbances of microbial colonization may be caused by various early life conditions which in turn may affect robustness of the chick in the long term. The aim of this thesis was to assess the effects of several early life factors including time of access to feed post hatch (immediately or 72 hours delayed), housing conditions, antibiotic treatment, and intestinal pathology on the intestinal microbiota composition, immune development, and specific antibody response later in life in chickens. Additionally, possible differences between broilers and layers were taken into account as unintentional co-selection of immunological traits may have taken place during the selection process for different production traits. Delayed access to feed and administration of antibiotics early in life led to a shift in early life microbiota composition, which seemed to be restored quite quickly in both cases. Microbiota composition in response to DSS was not investigated, but based on rodent studies was expected to be influenced. Ileal immune development, which was assessed in terms of relative cytokine and immunoglobulin mRNA expression levels was not affected by feeding strategy post hatch (early vs. delayed), but a downregulation of ileal immunoglobulin expression levels could be observed during DSS treatment. All early life factors investigated affected the specific antibody response towards an immunological challenge later in life. Interestingly, there seemed to be an interaction between immediate access to feed post hatch and immune responsiveness towards the environment, thus early feeding may influence the adaptive capacity of chickens in different environments. Regarding the differences between breeds it is interesting to note that broilers seem to have developed a more humoral oriented immune strategy, while layers seem to react in a more pro-inflammatory way. Taken together, results suggested that early life conditions may influence priming of the immune system during its developmental phase, leading to altered antibody responses later in life. Furthermore, broilers and layers seem to have developed different immune strategies. Early life conditions as well as possible differences between breeds should therefore be taken into account in future immunological studies.



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# CHAPTER 1

## General Introduction



### **Introduction**

Immune development and immune functionality may be influenced by management procedures of commercial hatcheries such as delayed access to feed post hatch, and may further be influenced by factors such as early life antibiotic treatment, housing conditions, and intestinal pathologies. These factors very likely also affect the microbiota composition in early life. Since the gut microbiota is in particular responsible for activation and development of the host immune system, disturbances of microbial colonization during early life may have long-lasting consequences for host immunity and therefore robustness of chickens. The experiments conducted within the scope of this thesis therefore aimed to investigate the consequences of different early life factors that may influence microbial colonization of the gut on immune development and later life immunity in chickens. Additionally, possible immunological differences between broilers and layers that are selected for different production traits were investigated.

This chapter will describe why adequate microbial colonization early in life is considered important for gut and immune development and which challenges the newly hatched chick faces in this regard. Furthermore, possible immunological consequences of disturbances of early life microbial colonization and of selection for certain production traits will be described. Finally, the research that was performed within the scope of this thesis will be outlined.

### **Importance of microbial colonization for gut and immune development**

Development towards a healthy gut and functioning immune system requires adequate microbial colonization of the intestine. Studies in germ-free animals illustrate the importance of microbial colonization for gut development. The absence of microbial stimuli in these animals leads to an abnormal gut development, which amongst other things is characterized by a distention of the cecum (Wostmann and Bruckner-Kardoss, 1959), a reduced intestinal mucosal surface area (Gordon and Bruckner-Kardoss, 1961), and an underdeveloped vascular network of the villi (Stappenbeck et al., 2002). Microbial colonization not only stimulates gut development, but is also required for immune development due

to the constant interaction between the intestinal microbiota and the host immune system (Hill and Artis, 2010; Round and Mazmanian, 2010). Priming of the innate immune system requires microbial colonization (Clarke et al., 2010) and Peyer's patches and T cell subsets develop in response to microbial colonization, which furthermore stimulates the production of secretory IgA and the functioning of macrophages (Barman et al., 1997; Smith et al., 2007; Round and Mazmanian, 2009). Moreover, microbial colonization is important for the induction of regulatory T cells and for maintaining the balance between regulatory and effector T cells (Mazmanian et al., 2005; Hall et al., 2008; Ishikawa et al., 2008; Ivanov et al., 2008; Geuking et al., 2011), and influences B cell development (Moreau et al., 1978; Bos et al., 1987; He et al., 2007; Hapfelmeier et al., 2010). In chickens it was recently found that immune complexes consisting of maternal antibodies and gut bacteria drive B cell differentiation in the bursa and play a role in the induction of specific systemic IgM antibodies (Sonoda et al., 2013; Ekino et al., 2015) indicating the importance of microbial colonization for the development of humoral immunity in chickens. Since maternal antibodies reflect the infection history of the hen, immune development in the offspring may transgenerationally be influenced via this mechanism. Microbial colonization was also shown to enhance the number of IgA and IgG secreting cells in various lymphoid tissues (Bos et al., 1988) as well as serum IgG levels (Gustafsson and Laurell, 1958), and leads to higher specific antibody responses (Ohwaki et al., 1977). Additionally, microbial colonization can decrease susceptibility to various bacterial infections (Taylor et al., 1961; Inagaki et al., 1996).

That a certain complexity in microbiota composition is necessary for immune activation has been shown in a study of Moreau et al. (1978), in which the combination of several, individually not stimulatory bacterial strains, led to an increase in intestinal IgA<sup>+</sup> cells. Immunomodulatory effects of intestinal bacteria have for instance been found for certain lactobacilli, which were shown to be able to enhance innate and adaptive immunity (Gill et al., 2000; Kawase et al., 2012), direct T cell mediated immune responses (Mohamadzadeh et al., 2005), and attenuate inflammatory processes in the gut (Schultz et al., 2002; Osman et al., 2004; Pathmakanthan et al., 2004; Petrof et al., 2009). Furthermore a combination of different clostridia was found to play an important role in the induction of

regulatory T cells, and in this way is important for the maintenance of intestinal homeostasis (Atarashi et al., 2011; Lopetuso et al., 2013).

Due to the described interaction between intestinal microbiota and the host immune system, disturbances of microbial colonization may have far-reaching consequences for host health.

### **Challenges faced by the newly hatched chick**

At hatch the chick's immune system is still immature and will gradually develop during the first weeks of life (Jeurissen et al., 1989). In first instance protection is provided via passive immunity by maternal antibodies present in the egg yolk (Kowalczyk et al., 1985; Kaspers et al., 1996; Hamal et al., 2006). Protection by maternal antibodies continuously subsides until about two weeks post hatch (Grindstaff et al., 2003; Hamal et al., 2006) and the chick has to rely on its own immune system. This period of dependency on passive immunity while the own adaptive immune system gradually develops has been proposed as a critical window in immune development (Butler and Šinkora, 2007). One of the most important sites from an immunological point of view is the gut and as described above, development towards a healthy gut and functioning immune system requires microbial colonization of the intestine, since there is a constant interaction between the intestinal microbiota and the immune system, which directs immune responses of the host (e.g. Hill and Artis, 2010; Round and Mazmanian, 2010). Placental mammals usually acquire their first microbes from their mother by passing through the birth canal (Dominguez-Bello et al., 2011). The chick on the other hand comes into contact with microbes present on the eggshell during the process of hatching, but bacteria may also enter the egg via pores in the eggshell (Berrang et al., 1999). In commercial hatcheries, however, transfer of microbes from hen to chick is largely prevented by disinfection of eggs and chicks. Chicks therefore have to rely on environmental microbes for colonization of the gut. Hereby bacteria present in feed play an important role and feed furthermore provides nutrients for the developing microbiota. In most cases, however, feed is not available in the immediate post hatch period. Despite some recent efforts being made to provide feed immediately post hatch by new hatching systems and on-farm hatching, the

vast majority of chicks still hatches in conventional hatching systems with no access to feed or water in the immediate post hatch period. The delay in access to feed may last up to 72 hours due to fixed management procedures at hatcheries. Chicks hatch over a period of 24 to 36 hours, but pulling of chicks in commercial hatcheries is set at a fixed time point of 21.5 days of incubation, a time point at which the majority of chicks has already hatched. Subsequent selection, counting, vaccination, and transport of chicks to the farm leads to a further delay in access to feed. This delay in access to feed and therefore colonization with a complex microbiota may have far-reaching consequences for chick (immune) development, especially since colonization is hampered in a period in which oral tolerance is developed. It has been shown that in the chicken oral tolerance, i.e. tolerance towards antigens present in feed, can only reliably be induced in the first 3 days post hatch under repeated antigenic stimulation (Klipper et al., 2000). Development of oral tolerance is important as it does not only lead to tolerance of antigens at mucosal sites, but also influences systemic immune responses (Pabst and Mowat, 2012). Failure to develop oral tolerance may result in hypersensitivity responses towards innocuous food antigens and immune homeostasis may be at stake (Pabst and Mowat, 2012). Apart from oral tolerance towards food antigens, development of mucosal tolerance towards harmless commensal bacteria is important in order to prevent intestinal pathologies due to hypersensitivity responses towards these bacteria (Tlaskalová-Hogenová et al., 2002; Pabst and Mowat, 2012). It is possible that, like oral tolerance, the reliable induction of mucosal tolerance is confined to the first 3 days post hatch as well and in that case chicks that face a long delay from the moment of hatch to their first intake of feed might be more prone to develop intestinal pathologies later in life.

### **Disturbance of early life microbial colonization and its consequences**

As mentioned above, microbial colonization is essential for development of a well-functioning immune system and especially microbial colonization early in life seems to be important in this regard.

In humans for instance the mode of delivery determines with which bacteria the newborn is initially colonized and differences in fecal microbiota composition

between children born vaginally or via caesarean section may persist for months (Grönlund et al., 1999; Huurre et al., 2008). While the former are colonized by microbiota found in the birth canal, the latter are colonized by microbiota found on the skin (Dominguez-Bello et al., 2010). Microbiota composition may further be influenced by the use of antibiotics and the type of infant feeding (Penders et al., 2006). Differences in initial microbiota composition may have long-lasting effects on immune development and therefore host health. It has been shown that in children delivered by caesarean section the number of IgM, IgG, and IgA secreting lymphocytes derived from blood is decreased during the first year of life compared with children delivered vaginally (Huurre et al., 2008). Furthermore children delivered by caesarean section are more prone to develop atopic disorders later in life (Kero et al., 2002; Bager et al., 2003; Renz-Polster et al., 2005; Salam et al., 2006). The same is true for children in which antibiotic treatment in the perinatal period led to a shift in microbiota composition (Farooqi and Hopkin, 1998; Wickens et al., 1999; Penders et al., 2007; Russell et al., 2013). Similar results have been found in rodents, in which germ-free mice showed a shift towards Th2 responses upon oral administration of an antigen, which was prevented when mice were conventionalized (Sudo et al., 1997). Conventionalization was, however, only effective when it took place in the neonatal period and remained ineffective at later ages (Sudo et al., 1997). Similarly, the administration of antibiotics leads to a shift towards Th2 responses and lower numbers of lymphocytes in spleen and Peyer's patches of mice only when antibiotics are administered early in life (Okayama et al., 2001). In accordance with the study of Okayama et al. (2001), antibiotics were found to increase the probability to develop allergic disorders only when antibiotics were administered to neonatal mice, but not when administered to adult mice (Russell et al., 2012). A study by Olszak et al. (2012) showed that invariant natural killer T cells, which form a bridge between the innate and adaptive arm of the immune system (Van Kaer et al., 2011), accumulate in mucosal tissues of germ-free mice, which subsequently leads to an enhanced morbidity in experimental models of allergic asthma and inflammatory bowel disease. Accumulation of invariant natural killer T cells in mucosal tissues and the development of pathologies could be prevented when germ-free animals were colonized with bacteria during neonatal life. Bacterial colonization did, however, not have a protective effect when colonization took place in adult life. It has been suggested

that the initial microbiota composition may be determinant for immune responses later in life, regardless of the adult microbiota (Maynard et al., 2012) and that the early life forms a critical window for immune development (Renz et al., 2012). Adequate microbial colonization early in life becomes even more important considering that the microbiota are not only important for gut and immune development, but also for the development of several other systems of the body, such as the respiratory, vascular, and endocrine system (Smith et al., 2007).

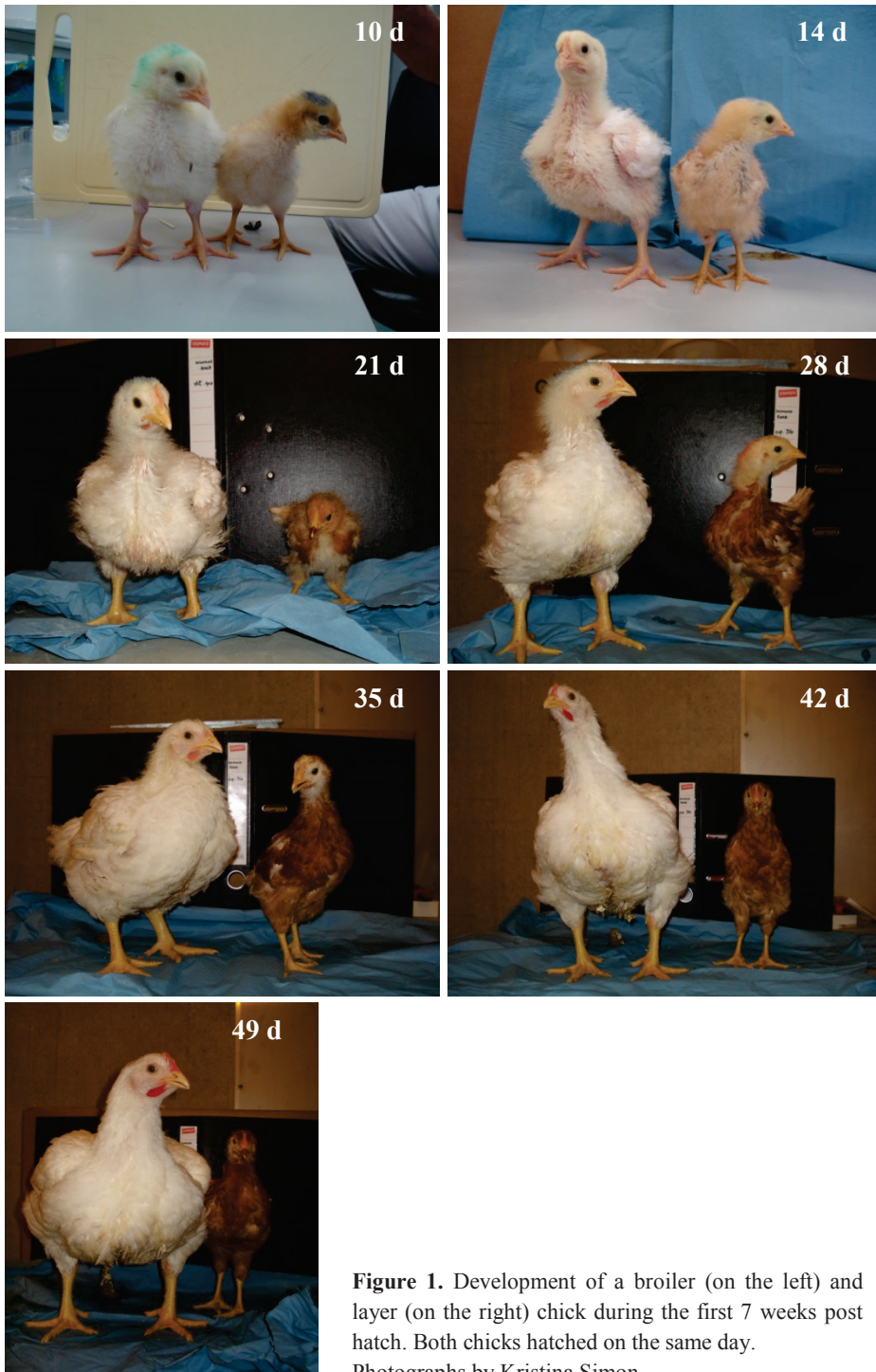
In chickens that are not able to be colonized with maternal microbiota in commercial hatcheries, feed probably plays an important part in microbial colonization of the gastro-intestinal tract and antigens present in feed may also directly interact with the gut immune system. A dramatic increase in bacterial numbers in the chicken intestine can be observed after the first ingestion of feed (Shapiro and Sarles, 1949). As mentioned before, especially early hatchers face a long delay until first feed intake in commercial hatcheries and it is very likely that this delay in access to feed leads to differences in early life microbiota composition between early hatchers that face a long delay and late hatchers that are relatively early fed. Consequently this delay in feed intake may have detrimental effects on gut and immune development. The first days post hatch seem to be particularly important for the development of the small intestine, where immune maturation and education takes place, and in early fed chicks the most dramatic development occurs within the first 24 hours post hatch (Geyra et al., 2001). Delayed fed chicks on the other hand lag behind in mucosal and villus development and show a reduced intestinal surface area (Uni et al., 1998; Noy et al., 2001; Lamot et al., 2014), a delayed appearance of germinal centers in the cecal tonsils, and a decreased weight of the bursa (Dibner et al., 1998; Bar-Shira et al., 2005). Antigenic stimulation of the bursa is important for B cell differentiation and therefore humoral immunity (Ekino et al., 1980; Ekino et al., 1985; Sonoda et al., 2013; Ekino et al., 2015) and a delayed access to feed post hatch may entail a delayed colonization of the hindgut and bursa with B and T lymphocytes (Bar-Shira et al., 2005). In accordance with these findings, delayed fed chicks show lower primary antibody responses to a model antigen in the first two weeks post hatch (Bar-Shira et al., 2005) and were reported to have a decreased response in the first three weeks post hatch to an orally administered coccidiosis vaccine which was used as a model for non-specific disease challenges (Dibner et al., 1998).

Taken together, microbial colonization in the neonatal period seems to be of great importance for (immune) development and disturbances in microbial colonization may have long-term consequences for immune reactivity and consequently host health. Early feeding studies performed in chickens so far focused on the first two to three weeks post hatch and it would be interesting to investigate the long-term effects of disturbances in microbial colonization on host immunity.

### **Differences between breeds**

Apart from differences in early life microbial colonization, genetic differences between breeds may have an influence on immune responses and therefore selection for different production traits may have had consequences for immune responses in different chicken breeds. Examples that selection for a certain trait often affects other traits as well come from domestication experiments, in which selection for tameness also affected morphological traits such as body size, coat color, and ear shape, and physiological processes such as stress responsiveness and reproductive cycle (Trut et al., 2009). Over the last decades a strong selection for certain production traits has taken place in chickens (Figure 1). Broilers were selected for maximal meat production in a minimal amount of time and while a broiler in 1957 weighed on average 905 g at day 56, by 2005 its average weight had increased to an enormous 4202 g at day 56 (Zuidhof et al., 2014). Layers on the other hand were selected for maximal egg production and while a laying hen at the end of the 19<sup>th</sup> century produced between 80 and 85 eggs a year, the average egg production had increased to more than 300 eggs per year by 2014 (CBS, 2014).

Various studies indicate that selection for a certain production trait has also affected immunity in chickens. Selection for increased bodyweight in broilers and turkeys or increased egg production in turkeys leads to a decrease in the relative weight of primary and secondary immune organs (Bayyari et al., 1997; Cheema et al., 2003), and a decrease in disease resistance in these birds (Yunis et al., 2000; Huff et al., 2005). It cannot be excluded that selection for increased egg production in layers has had effects on immune parameters similar to those observed in turkeys. Furthermore, selection for increased bodyweight in broilers and layers



**Figure 1.** Development of a broiler (on the left) and layer (on the right) chick during the first 7 weeks post hatch. Both chicks hatched on the same day. Photographs by Kristina Simon.

leads to a decrease in specific antibody responses (Miller et al., 1992; Qureshi and Havenstein, 1994; Cheema et al., 2003) and the period in which elevated antibody levels are maintained after an immunological challenge is shortened (Miller et al., 1992). On the other hand cell-mediated and inflammatory responses were reported to be increased by selection for high bodyweight (Cheema et al., 2003), while other parts of the immune system, such as macrophage and NK cell functions seemed to be unaffected (Qureshi and Havenstein, 1994). Interestingly, immune responses seem to be more affected by selection for bodyweight than by selection for egg production, since disease resistance was decreased to a larger extent in birds selected for high bodyweight, which also showed less pronounced cutaneous hypersensitivity responses compared with birds selected for high egg production (Bayyari et al., 1997; Huff et al., 2005).

Most studies investigate the consequences of selection for different production traits within one breed, but some researchers also investigated differences between broilers and layers. Leshchinsky and Klasing (2001) reported lower pro-inflammatory cytokine mRNA expression levels in spleen cells of broilers compared with layers, which may indicate a difference in macrophage activity between breeds. Differences between breeds in cellular responses in a spleen cell proliferation assay depended on the mitogen used and the response was lower for LPS, but higher for concanavalin A and phytohemagglutinin in broilers compared with layers (Leshchinsky and Klasing, 2001). Koenen et al. (2002) reported a lack of specific cellular responses to trinitrophenyl-conjugated keyhole limpet hemocyanin and weaker non-specific cellular responses to concanavalin A in a spleen and peripheral blood lymphocyte proliferation assay in broilers compared with layers. With respect to the specific antibody response, broilers in that study showed higher IgM and lower IgY antibody responses compared with layers and maintained their elevated antibody levels for a shorter period of time than layers.

Taken together, selection for specific production traits seems to have influenced immunity in chickens. Selection for production traits is ongoing, and so is probably the potentially unintentional selection for immunological traits and its consequences for host health.

## Concluding remarks and outline of this thesis

Taken together, adequate microbial colonization during the neonatal period is essential for immune development and although in recent years efforts have been made to provide immediate access to feed post hatch by either availability of feed in the hatching cabinet or by on-farm hatching systems, the majority of chicks still hatches in conventional hatching cabinets without access to feed or water. Delayed access to feed which leads to a delay in initial microbial colonization, and other factors that disturb microbiota composition early in life, such as antibiotic treatment or intestinal pathologies, may have far-reaching consequences for immune development, and consequently immune competence and disease resistance later in life. Additionally, selection for different production traits may have led to a (unintentional) co-selection of immunological traits, so that disturbance of early life microbial colonization may have a different effect on later life immunity in broilers than it does in layers.

The research performed within the scope of this thesis therefore investigated different early life conditions and interventions that may affect immunity later in life by disturbing early life microbial colonization. In that respect differences between broilers and layers were taken into account. In a first study, the effect of immediate or delayed access to feed on ileal immune development in broilers and layers was investigated (chapter 2). For this purpose broiler and layer chicks either received immediate access to feed post hatch or with a 72 hour delay. Ileal samples from both breeds and treatment groups were obtained over time and relative ileal mRNA expression levels were determined for a selection of pro- and anti-inflammatory cytokines as well as immunoglobulins in order to gain insight in the dynamics of ileal immune development in the two breeds.

In a second study, the effects of early and delayed feeding on the specific antibody response later in life were investigated in broilers kept in different housing systems, since early feeding strategy might influence a bird's sensitivity towards its environment (chapter 3). Again chicks either received immediate access to feed post hatch or with a 72 hour delay and chicks of both feeding strategies were either housed in a floor system containing wood shavings or in a cage system. At 4 weeks of age birds received a non-infectious lung challenge consisting of a combination of *E. coli* derived lipopolysaccharide (LPS) or human serum albumin

(HuSA). Control birds received phosphate buffered saline. The birds' sickness response was assessed in the hours following administration of the challenge and specific antibody titers were determined over the course of two weeks.

As antibiotic treatment influences the intestinal microbiota and may have long-term consequences on host immunity, in a third study one-day old layer chicks were administered broad spectrum antibiotics during the first week of life, followed by milder antibiotic treatment during the following two weeks (chapter 4). Control birds did not receive any antibiotics. Fecal microbiota composition was determined during and after cessation of antibiotic treatment. At 15 weeks of age, i.e. 12 weeks after cessation of antibiotic treatment, all birds received a non-infectious lung challenge consisting of a combination of LPS and HuSA and specific antibody titers were determined 10 days after administration of the LPS/HuSA challenge.

In a final experiment, the effect of early life intestinal pathologies on ileal immune development and the specific antibody response later in life was investigated in early fed broilers and layers (chapter 5). Administration of dextran sulfate sodium (DSS), which is widely used in rodent models of inflammatory bowel disease, was adapted as a model for intestinal pathology in chickens. DSS was administered via drinking water to chicks of both breeds between day 11 and day 18 post hatch. Control birds received plain drinking water. At day 35 an LPS/HuSA challenge was administered intramuscularly to all chickens and specific antibody titers were determined over the course of two weeks.

In the final chapter of this thesis (chapter 6), the findings of the studies described above will be integrated and implications of these results as well as perspectives for future research will be discussed.

## REFERENCES

- Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, Y. Tanigushi, K. Takeda, S. Hori, I. I. Ivanov, Y. Umesaki, K. Itoh, and K. Honda. 2011. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* 331:337-341.
- Bager, P., M. Melbye, K. Rostgaard, C. Stabell Benn, and T. Westergaard. 2003. Mode of delivery and risk of allergic rhinitis and asthma. *J. Allergy Clin. Immunol.* 111:51-56.
- Bar-Shira, E., D. Sklan, and A. Friedman. 2005. Impaired immune responses in broiler hatchling hindgut following delayed access to feed. *Vet. Immunol. Immunopathol.* 105:33-45.
- Barman, N. N., A. T. J. Bianchi, R. J. Zwart, R. Pabst, and H. J. Rothkötter. 1997. Jejunal and ileal Peyer's patches in pigs differ in their postnatal environment. *Anat. Embryol.* 195:41-50.
- Bayyari, G. R., W. E. Huff, N. C. Rath, J. M. Balog, L. A. Newberry, J. D. Villines, J. K. Skeeles, N. B. Anthony, and K. E. Nestor. 1997. Effect of the genetic selection of turkeys for increased body weight and egg production on immune and physiological responses. *Poult. Sci.* 76:289-296.
- Berrang, M. E., N. A. Cox, J. F. Frank, and R. J. Buhr. 1999. Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: a review. *J Appl Poult Res* 8:499-504.
- Bos, N. A., C. G. Meeuwssen, H. Hooijkaas, R. Benner, B. S. Wostmann, and J. R. Pleasants. 1987. Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. *Cell. Immunol.* 105:235-245.
- Bos, N. A., C. G. Meeuwssen, B. S. Wostmann, J. R. Pleasants, and R. Benner. 1988. The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. *Cell. Immunol.* 112:371-380.
- Butler, J. E., and M. Šinkora. 2007. The isolator piglet: a model for studying the development of adaptive immunity. *Immunol. Res.* 39:33-51.
- CBS. 2014. Centraal Bureau voor Statistiek. <http://www.cbs.nl/nl-NL/menu/themas/dossiers/historische-reeksen/publicaties/artikelen/archief/2014/2014-kippen-historie-2013-wm.htm>.

- Cheema, M. A., M. A. Qureshi, and G. B. Havenstein. 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* 82:1519-1529.
- Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16:228-231.
- Dibner, J. J., C. D. Knight, M. L. Kitchell, C. A. Atwell, A. C. Downs, and F. J. Ivey. 1998. Early feeding and development of the immune system in neonatal poultry. *J Appl Poult Res* 7:425-436.
- Dominguez-Bello, M. G., M. J. Blaser, R. E. Ley, and R. Knight. 2011. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology* 140:1713-1719.
- Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. U.S.A.* 107:11971-11975.
- Ekino, S., Y. Nawa, K. Tanaka, K. Matsuno, H. Fuji, and M. Kotani. 1980. Suppression of immune response by isolation of the bursa of Fabricius from environmental stimuli. *Aust J Exp Biol Med Sci* 58:289-296.
- Ekino, S., K. Sonoda, and S. Inui. 2015. Origin of IgM<sup>+</sup>IgG<sup>+</sup> lymphocytes in the bursa of Fabricius. *Cell Tissue Res.* 362:153-162.
- Ekino, S., K. Suginozono, T. Urano, H. Fuji, K. Matsuno, and M. Kotani. 1985. The bursa of Fabricius: a trapping site for environmental antigens. *Immunology* 55:405-410.
- Farooqi, I. S., and J. M. Hopkin. 1998. Early childhood infection and atopic disorder. *Thorax* 53:927-932.
- Geuking, M. B., J. Cahenzli, M. A. E. Lawson, D. C. K. Ng, E. Slack, S. Hapfelmeier, K. D. McCoy, and A. J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 34:794-806.
- Geyra, A., Z. Uni, and D. Sklan. 2001. Enterocyte dynamics and mucosal development in the posthatch chick. *Poult. Sci.* 80:776-782.
- Gill, H. S., K. J. Rutherford, J. Prasad, and P. K. Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Br. J. Nutr.* 83:167-176.
- Gordon, H. A., and E. Bruckner-Kardoss. 1961. Effect of normal microbial flora on intestinal surface area. *Am. J. Physiol.* 1:175-178.

- Grindstaff, J. L., E. D. Brodie III, and E. D. Ketterson. 2003. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proc. R. Soc. Lond., B, Biol. Sci.* 270:2309-2319.
- Grönlund, M.-M., O.-P. Lehtonen, E. Eerola, and P. Kero. 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J. Pediatr. Gastroenterol. Nutr.* 28:19-25.
- Gustafsson, B. E., and C. B. Laurell. 1958. Gamma globulins in germ-free rats. *J. Exp. Med.* 108:251-258.
- Hall, J. A., N. Bouladoux, C. M. Sun, E. A. Wohlfert, R. B. Blank, Q. Zhu, M. E. Grigg, J. A. Berzofsky, and Y. Belkaid. 2008. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity* 29:637-649.
- Hamal, K. R., S. C. Burgess, I. Y. Pevzner, and G. F. Erf. 2006. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult. Sci.* 85:1364-1372.
- Häpfelmeier, S., M. A. E. Lawson, E. Slack, J. K. Kirundi, M. Stoel, M. Heikenwalder, J. Cahenzli, Y. Velykoredko, M. L. Balmer, K. Endt, M. B. Geuking, R. Curtiss, K. D. McCoy, and A. J. Macpherson. 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* 328:1705-1709.
- He, B., W. Xu, P. A. Santini, A. D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D. M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. *Immunity* 26:812-826.
- Hill, D. A., and D. Artis. 2010. Intestinal bacteria and the regulation of immune cell homeostasis. *Annu. Rev. Immunol.* 28:623-667.
- Huff, G. R., W. E. Huff, J. M. Balog, N. C. Rath, N. B. Anthony, and K. E. Nestor. 2005. Stress response differences and disease susceptibility reflected by heterophil to lymphocyte ratio in turkeys selected for increased body weight. *Poult. Sci.* 84:709-717.
- Huurre, A., M. Kalliomäki, S. Rautava, M. Rinne, S. Salminen, and E. Isolauri. 2008. Mode of delivery - effects on gut microbiota and humoral immunity. *Neonatology* 93:236-240.
- Inagaki, Y., T. Suzuki, K. Nomoto, and Y. Yoshikai. 1996. Increased susceptibility to primary infection with *Listeria monocytogenes* in germfree mice may be due to

- lack of accumulation of L-selectin<sup>+</sup> CD44<sup>+</sup> T cells in sites of inflammation. *Infect. Immun.* 64:3280-3287.
- Ishikawa, H., K. Tanaka, Y. Maeda, Y. Aiba, A. Hata, N. M. Tsuji, Y. Koga, and T. Matsumoto. 2008. Effect of intestinal microbiota on the induction of regulatory CD25<sup>+</sup> CD4<sup>+</sup> T cells. *Clin. Exp. Immunol.* 153:127-135.
- Ivanov, I. I., R. de Llanos Frutos, N. Manel, K. Yoshinaga, D. B. Rifkin, R. Balfour Sartor, B. Brett Finlay, and D. R. Littman. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4:337-349.
- Jeurissen, S. H. M., E. M. Janse, G. Koch, and G. F. De Boer. 1989. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* 258:119-124.
- Kaspers, B., H. Bondl, and T. W. F. Göbel. 1996. Transfer of IgA from albumen into the yolk sac during embryonic development in the chicken. *J. Vet. Med. A.* 43:225-231.
- Kawase, M., F. He, A. Kubota, K. Yoda, K. Miyazawa, and M. Hiramatsu. 2012. Heat-killed *Lactobacillus gasseri* TMC0356 protects mice against influenza virus infection by stimulating gut and respiratory immune responses. *FEMS Immunol. Med. Microbiol.* 64:280-288.
- Kero, J., M. Gissler, M.-M. Grönlund, P. Kero, P. Koskinen, E. Hemminki, and E. Isolauri. 2002. Mode of delivery and asthma - Is there a connection? *Pediatr. Res.* 52:6-11.
- Klipper, E., D. Sklan, and A. Friedman. 2000. Immune responses of chickens to dietary protein antigens I. Induction of systemic and intestinal immune responses following oral administration of soluble proteins in absence of adjuvant. *Vet. Immunol. Immunopathol.* 74:209-223.
- Koenen, M. E., A. G. Boonstra-Blom, and S. H. M. Jeurissen. 2002. Immunological differences between layer- and broiler-type chickens. *Vet. Immunol. Immunopathol.* 89:47-56.
- Kowalczyk, K., J. Daiss, J. Halpern, and T. F. Roth. 1985. Quantitation of maternal-fetal IgG transport in the chicken. *Immunology* 54:755-762.
- Lamot, D., I. B. Van De Linde, R. Molenaar, C. W. Van der Pol, P. J. A. Wijtten, B. Kemp, and H. Van Den Brand. 2014. Effects of moment of hatch and feed access on chicken development. *Poult. Sci.* 93:1-11.
- Leshchinsky, T. V., and K. C. Klasing. 2001. Divergence of the inflammatory response in two types of chickens. *Dev. Comp. Immunol.* 25:629-638.
- Lopetuso, L. R., F. Scaldaferri, V. Petito, and A. Gasbarrini. 2013. Commensal clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathogens* 5:23.

- Maynard, C. L., C. O. Elson, R. D. Hatton, and C. T. Weaver. 2012. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489:231-241.
- Mazmanian, S. K., C. H. Liu, A. O. Tzianabos, and D. L. Kasper. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122:107-118.
- Miller, L. L., P. B. Siegel, and E. A. Dunnington. 1992. Inheritance of antibody response to sheep erythrocytes in lines of chickens divergently selected for fifty-six-day body weight and their crosses. *Poult. Sci.* 71:47-52.
- Mohamadzadeh, M., S. Olson, W. V. Kalina, G. Ruthel, G. L. Demmin, K. L. Warfield, S. Bavari, and T. R. Klaenhammer. 2005. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc. Natl. Acad. Sci. U.S.A.* 102:2880-2885.
- Moreau, M. C., R. Ducluzeau, D. Guy-Grand, and M. C. Muller. 1978. Increase in the Population of Duodenal Immunoglobulin A Plasmocytes in Axenic Mice Associated with Different Living or Dead Bacterial Strains of Intestinal Origin. *Infect. Immun.* 21:532-539.
- Noy, Y., A. Geyra, and D. Sklan. 2001. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poult. Sci.* 80:912-919.
- Ohwaki, M., N. Yasutake, H. Yasui, and R. Ogura. 1977. A comparative study on the humoral immune response in germ-free and conventional mice. *Immunology* 32:43-48.
- Okayama, N., N. Sudo, H. Sogawa, and C. Kubo. 2001. Antibiotic use during infancy promotes a shift in the  $T_H1/T_H2$  balance toward  $T_H2$ -dominant immunity in mice. *J. Allergy .Clin. Immunol.* 107:153-159.
- Olszak, T., D. An, S. Zeissig, M. Pinilla Vera, J. Richter, A. Franke, J. N. Glickman, R. Siebert, R. M. Baron, D. L. Kasper, and R. S. Blumberg. 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336:489-493.
- Osman, N., D. Adawi, S. Ahrne, B. Jeppsson, and G. Molin. 2004. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of *Lactobacillus* and *Bifidobacterium*. *Dig. Dis. Sci.* 49:320-327.
- Pabst, O., and A. M. Mowat. 2012. Oral tolerance to food protein. *Nature* 5:232-239.
- Pathmakanthan, S., C. K. F. Li, J. Cowie, and C. J. Hawkey. 2004. *Lactobacillus plantarum* 299: beneficial in vitro immunomodulation in cells extracted from inflamed human colon. *J. Gastroenterol. Hepatol.* 19:166-173.
- Penders, J., C. Thijs, P. A. Van den Brandt, I. Kummeling, B. Snijders, F. Stelma, H. Adams, R. Van Ree, and E. E. Stobberingh. 2007. Gut microbiota composition and

- development of atopic manifestations in infancy: the KOALA birth cohort study. *Gut* 56:661-667.
- Penders, J., C. Thijs, C. Vink, F. F. Stelma, B. Snijders, I. Kummeling, P. A. Van den Brandt, and E. E. Stobberingh. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 118:511-521.
- Petrof, E. O., E. C. Claud, J. Sun, T. Abramova, Y. Guo, T. S. Waypa, S.-M. He, Y. Nakagawa, and E. B. Chang. 2009. Bacteria-free solution derived from *Lactobacillus plantarum* inhibits multiple NF-KappaB pathways and inhibits proteasome function. *Inflamm. Bowel Dis.* 15:1537-1547.
- Qureshi, M. A., and G. B. Havenstein. 1994. A comparison of the immune performance of a 1991 commercial broiler with a 1957 randombred strain when fed "typical" 1957 and 1991 broiler diets. *Poult. Sci.* 73:1805-1812.
- Renz-Polster, H., M. R. David, A. S. Buist, W. M. Vollmer, E. A. O'Connor, E. A. Frazier, and M. A. Wall. 2005. Caesarean section delivery and the risk of allergic disorders in childhood. *Clin. Exp. Allergy* 35:1466-1472.
- Renz, H., P. Brandtzaeg, and M. Hornef. 2012. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nat. Rev. Immunol.* 12:9-23.
- Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9:313-323.
- Round, J. L., and S. K. Mazmanian. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc. Natl. Acad. Sci. U.S.A.* 107:12204-12209.
- Russell, S. L., M. J. Gold, M. Hartmann, B. P. Willing, L. Thorson, M. Wlodarska, N. Gill, M.-R. Blanchet, W. W. Mohn, K. M. McNagny, and B. B. Finlay. 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep.* 13:440-447.
- Russell, S. L., M. J. Gold, B. P. Willing, L. Thorson, K. M. McNagny, and B. B. Finlay. 2013. Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. *Gut Microbes* 4:158-164.
- Salam, M. T., H. G. Margolis, R. McConnell, J. A. McGregor, E. L. Avol, and F. D. Gilliland. 2006. Mode of delivery is associated with asthma and allergy occurrences in children. *Ann Epidemiol* 16:341-346.
- Schultz, M., C. Veltkamp, L. A. Dieleman, W. B. Grenther, P. B. Wyrick, L. Tonkonogy, and R. B. Sartor. 2002. *Lactobacillus plantarum* 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm. Bowel Dis.* 8:71-80.

- Shapiro, S. K., and W. B. Sarles. 1949. Microorganisms in the intestinal tract of normal chickens. *J. Bacteriol.* 58:531-544.
- Smith, K., K. D. McCoy, and A. J. Macpherson. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* 19:59-69.
- Sonoda, K., K. Noguchi, and S. Ekino. 2013. Immune complexes of *E. coli* antigens and maternal IgG in the bursa of Fabricius. *Cell Tissue Res.* 354:813-821.
- Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:15451-15455.
- Sudo, N., S. Sawamura, K. Tanaka, C. Kubo, and Y. Koga. 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J. Immunol.* 159:1739-1745.
- Taylor, M. J., J. R. Rooney, and G. P. Blundell. 1961. Experimental anthrax in the rat: II. The relative lack of natural resistance in germ-free (lobund) hosts. *Am. J. Pathol.* 38:625-638.
- Tlaskalová-Hogenová, H., L. Tučková, R. Lodinová-Žádníková, R. Štěpánková, B. Cukrowska, D. P. Funda, I. Stříž, H. Kozáková, I. Trebichavský, D. Sokol, Z. Řeháková, J. Šinkora, P. Fundová, D. Horáková, L. Jelínková, and D. Sánchez. 2002. Mucosal immunity: its role in defense and allergy. *Int. Arch. Allergy Immunol.* 128:77-89.
- Trut, L., I. Oskina, and A. Kharlamova. 2009. Animal evolution during domestication: the domesticated fox as a model. *Bioessays* 31:349-360.
- Uni, Z., S. Ganot, and D. Sklan. 1998. Posthatch development of mucosal function in the broiler small intestine. *Poult. Sci.* 77:75-82.
- Van Kaer, L., V. V. Parekh, and L. Wu. 2011. Invariant natural killer T cells: bridging innate and adaptive immunity. *Cell Tissue Res.* 343:43-55.
- Wickens, K., N. Pearce, J. Crane, and R. Beasley. 1999. Antibiotic use in early childhood and the development of asthma. *Clin. Exp. Allergy* 29:766-771.
- Wostmann, B., and E. Bruckner-Kardoss. 1959. Development of cecal distention in germ-free baby rats. *Am. J. Physiol.* 6:1345-1346.
- Yunis, R., A. Ben-David, E. D. Heller, and A. Cahaner. 2000. Immunocompetence and viability under commercial conditions of broiler groups differing in growth rate and in antibody response to *Escherichia coli* vaccine. *Poult. Sci.* 79:810-816.
- Zuidhof, M. J., B. L. Schneider, V. L. Carney, D. R. Korver, and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. *Poult. Sci.* 93:1-13.



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## **CHAPTER 2**

### **Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers**

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## ABSTRACT

Provision of feed in the immediate post-hatch period may influence interaction between intestinal microbiota and immune system, and consequently immunological development of the chick. This study addressed ileal immune development in response to early feeding in 2 chicken breeds selected for different production traits: broilers and layers. Chicks of both breeds either received feed and water immediately post-hatch or were subjected to a 72-h feed and water delay. Ileal cytokine and immunoglobulin mRNA expression levels were determined at different time points. Effects of early feeding were limited, but breeds differed strikingly regarding cytokine and immunoglobulin expression levels. Cytokine expression levels in broilers were low compared with layers and showed a transient drop in the second to third week of life. In contrast, broilers showed considerably higher expression levels of IgA, IgM, and IgY. These findings indicate that the 2 breeds use different immune strategies, at least on ileal level.

**Key words:** chicken, ileum, cytokine, immunoglobulin, gene expression

## INTRODUCTION

Immediately after initial feed intake, the chicken intestine is rapidly colonized by high numbers of bacteria (Shapiro and Sarles, 1949), and availability of feed in the immediate post hatch (**p.h.**) period has been shown to accelerate intestinal development (Uni et al., 1998; Noy et al., 2001), manifested as enhanced surface area and cell proliferation. More recently, an interplay between diet, intestinal microbiota, and immune system has been assumed (Round and Mazmanian, 2009; Turnbaugh et al., 2009; Maslowski and Mackay, 2011), because diet influences intestinal microbiota composition (Turnbaugh et al., 2009; De Filippo et al., 2010), which in turn influences immune development (Wen et al., 2008; Clarke et al., 2010). Therefore, the onset of provision of feed in the immediate p.h. period may also be important for immune development. The first provision of feed may be delayed for up to 72 h p.h. in modern husbandry because of differences in the time of hatch relative to removing all chicks from the incubator at d 21 and subsequent transport of chicks to the farm.

In early feeding (**EF**) studies, the importance of provision of feed in the immediate p.h. has been assessed from an immunological point of view. In broilers, EF has been reported to result in an increased weight of the bursa and improved response to disease challenges when compared with chicks from which food was withheld (Dibner et al., 1998; Bar Shira et al., 2005). Moreover, EF results in a more rapid colonization of hindgut and bursa with T and B lymphocytes, as well as more pronounced primary antibody responses (Bar Shira et al., 2005). In layers, however, the effects of EF on intestinal immune development are unknown.

Concerning immune development, the ileum is generally considered the site of immune activation. Ileal immune development has been assessed previously in broilers for the first 2 wk p.h. (Bar-Shira et al., 2003) and layers for the first 10 to 12 wk p.h. (Jeurissen et al., 1989; Lammers et al., 2010), but effects of EF were not taken into account in these studies. In layers an up-regulation of pro- [IL-1, IL-12p40, interferon (**IFN**) $\gamma$ ], and anti- [IL-10, transforming growth factor (**TGF**) $\beta$ ] inflammatory cytokine expression levels between 2 and 6 wk p.h. was found (Lammers et al., 2010). Moreover, IgA expression levels reached their maximum when cytokine expression levels returned to basal levels between wk 7 and 10. It

was hypothesized that IgA plays a role in maintenance of intestinal homeostasis and that the period of cytokine upregulation is a prerequisite for IgA production.

It can be questioned, however, whether those dynamics in immune development are also present in broilers. The aim of this study was therefore to extend the existing knowledge on ileal immune development in broilers as well as layers over a longer period of time in combination with 2 feeding strategies: early feeding (**EF**, immediately p.h.) or delayed feeding (**DF**, 72 h p.h.). Gaining insight in the processes of ileal immune development will help to define possible critical windows in immune development for both breeds. This knowledge may be useful for development of well-directed immune modulatory feeding strategies.

## **MATERIALS AND METHODS**

### ***Chickens and Housing***

In this study, 2 chicken breeds were used: fast-growing broilers (Ross 308, n = 150) and layers (Lohman Brown, n = 210). Eggs from both breeds were obtained from 2 commercial hatcheries (Lagerwey Hatchery, Lunteren, the Netherlands, and Verbeek Hatchery, Zeewolde, the Netherlands) and were incubated at our department under standard incubation conditions applying to each breed. From embryonic d 19 onward, eggs were checked regularly for hatching. Dry chicks were removed from the incubator every 3 h, color coded, and randomly assigned to 1 of 2 treatments: EF or DF. Chicks in the DF group had no access to feed and water during the first 72 h p.h. From 72 h p.h. onward, all chicks were given ad libitum access to feed and water. Feed for each breed consisted of its appropriate commercial starter diet containing a coccidiostat (salinomycin). Chicks were group-housed per breed in separate rooms in pens of 2 x 2 m containing wood shavings with a regimen of 16L:8D. At an age of 2 wk, all chicks received an obligatory Newcastle disease vaccination. There were 10 replicates per feeding treatment for each breed, and pen was considered the experimental unit.

This study was approved by the Animal Welfare Committee of Wageningen University and Research Centre in accordance with Dutch laws and regulations on the execution of animal experiments.

### ***Measurements and Sampling***

Chicks were weighed at hatch, d 3 and d 7, and from then onward once a week.

Ileum midsections of 1 cm between Meckel's diverticulum and ceco-iliac junction were collected at several time points. Time points were immediately p.h., and d 3, 6, 9, 14, 21, 35, and 42 p.h. for both breeds. Additional time points for layers were d 49, 70, and 140 p.h. Immediately p.h., 10 chicks were killed per breed. For all other time points 10 chicks were killed per breed and treatment. Tissue sections of 50 to 100 mg were stored overnight at 4°C in RNA later and subsequently stored at -80°C until use.

Furthermore broiler ileum samples for immunohistochemistry were collected immediately p.h., and d 3, 6, 9, 14, 21, 35, and 42 p.h. Swiss rolls were made out of 5-cm ileum samples situated adjacent to the midsection and were immediately fixed overnight in Carnoy's fixative consisting of 60% methanol, 30% chloroform, and 10% glacial acetic acid. After embedding in paraffin, sections of 7 µm were cut and stained immunohistochemically for IgA.

Blood was collected from each chicken before section for determination of IgM and IgY natural antibody (NAb) titers against keyhole limpet hemocyanin (KLH), because NAb are likely to contribute to the first line of defense against pathogens.

Weights of spleen and bursa relative to BW were determined during sections on d 6, 9, 14, 21, 35, 42, 49, 70, and 140 (n=10/treatment/d), because development of lymphoid organ weight may be a first indicator of immune development. Because broilers are usually slaughtered at an age of 6 wk, the last section for those animals occurred on d 42.

### ***RNA Isolation & cDNA Synthesis***

Total RNA was extracted from midsections of ileum using TRIzol Reagent (catalog 15596-026, Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. Concentrations of RNA were equaled by dilution in diethylpyrocarbonate-treated water after RNA concentration was measured by a spectrophotometer.

Prior to cDNA synthesis, DNase treatment was applied (all chemicals originated from Invitrogen, Carlsbad, CA). One microliter of DNase I (DNase I amplification grade kit, also including DNase I reaction buffer and 25 mM EDTA; catalog 18068-015) and 1  $\mu$ L of 10x DNaseI reaction buffer was added to 500 ng of total RNA and incubated for 15 min at room temperature (**RT**) at a total volume of 12  $\mu$ L. The DNase I was inactivated by adding 1  $\mu$ L 25 mM EDTA and incubation for 10 min at 65°C. After incubation, samples were quickly cooled down on ice.

For cDNA synthesis, 300 ng of Random Hexamer primers and 1  $\mu$ L of 10 mM dNTP (catalog 18427-013) was added to each sample and samples were incubated for 10 min at 70°C. After quickly cooling the samples on ice, 1  $\mu$ L RNase OUT (catalog 10777-019), 1  $\mu$ L Superscript II (SuperScript II Reverse Transcriptase kit, also including First Strand Buffer and DL-dithiothreitol, catalog 18064-014), 4  $\mu$ L 5x First Strand Buffer, and 2  $\mu$ L DL-dithiothreitol was added to each sample. Samples were then first incubated for 50 min at 37°C, followed by 10 min at 70°C. Samples were quickly cooled down on ice and stored at -20°C until use.

### ***Real-Time Quantitative PCR***

Real-time quantitative PCR was performed to obtain relative mRNA expression levels of pro-inflammatory cytokines IL-12p40, IL-1 $\beta$ , and IFN- $\gamma$ , and anti-inflammatory cytokines IL-10 and TGF- $\beta$ , as well as IgM, IgY, and IgA. The reference gene used was 28S. Primer sequences are listed in

1. Samples of cDNA were diluted 1:5,000 for 28S and 1:50 for the genes of interest. Primers were checked for amplification efficiency, which was between 1.9 and 2.1 for all primers. An end volume of 25  $\mu$ L per well was reached by adding 5  $\mu$ L of cDNA, 12.5  $\mu$ L of SYBR Green PCR Mastermix (catalog 4309155, Applied Biosystems, Warrington, UK), 1.25  $\mu$ L each of 10  $\mu$ M forward and reverse primer, and 5  $\mu$ L of demineralized water to each well.

Real-time quantitative PCR was performed on an Applied Biosystems 7500 Real Time PCR system. The real-time quantitative PCR protocol consisted of 10 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 59°C, 36 s at 72°C, 15 s at 95°C, and 1 min at 59°C. Melting curves were obtained after each run by detection of fluorescence at 1°C intervals from 60 to 90°C.

**Table 1.** Primer sequences

Gene	Accession number	Primer sequence <sup>1</sup> (5' → 3')
<b>28S</b>	DQ018756	F: GGC-GAA-GCC-AGA-GGA-AAC-T R: GAC-GAC-CGA-TTT-GCA-CGT-C
<b>IL-1β</b>	AJ245728	F: CAG-CAG-CCT-CAG-CGA-AGA-G R: CTG-TGG-TGT-GCT-CAG-AAT-CCA
<b>IL-10</b>	AJ621614	F: CGC-TGT-CAC-CGC-TTC-TTC-A R: TCC-CGT-TCT-CAT-CCA-TCT-TCT-C
<b>IL-12p40</b>	NM_213571.1	F: GAC-CCA-CGA-GAT-TAT-CAG-CTA-CAG-T R: TGC-TTG-GCT-CTT-TAT-AGC-TTT-TCA
<b>INF-γ</b>	Y07922	F: GTG-AAG-AAG-GTG-AAA-GAT-ATC-ATG-GA R: GCT-TTG-CGC-TGG-ATT-CTC-A
<b>TGF-β</b>	M31160	F: ACC-TCG-ACA-CCG-ACT-ACT-GCT-T R: ATC-CTT-GCG-GAA-GTC-GAT-GT
<b>IgM</b>	X01613.1	F: GCA-TCA-GCG-TCA-CCG-AAA-GC R: TCC-GCA-CTC-CAT-CCT-CTT-GC
<b>IgY</b>	X07174.1	F: ATC-ACG-TCA-AGG-GAT-GCC-CG R: ACC-AGG-CAC-CTC-AGT-TTG-G
<b>IgA</b>	S40610	F: GTC-ACC-GTC-ACC-TGG-ACT-ACA R: ACC-GAT-GGT-CTC-CTT-CAC-ATC

<sup>1</sup>F = forward; R = reverse

Relative expression ratios of target genes were calculated according to the method described by Pfaffl (2001), accounting for an internal control (28S) as well as an external calibrator (d 0 as untreated control).

Onset of immunoglobulin and cytokine expression levels was defined as the time point at which the respective expression levels exceeded those of d 0 significantly, and this time point was regarded as a sign of the start of immune maturation. Dynamics in expression levels are described in a descriptive way.

### ***Immunohistochemistry***

For immunohistochemical staining for IgA, sections were deparaffinized, rehydrated, and endogenous peroxidase was quenched with methanol containing 0.5% hydrogen peroxide. After washing with PBS containing 0.1% Triton X-100 (**PBS-t**) sections were incubated with 10% normal horse serum to diminish

background and unspecific staining. Goat-anti-chicken IgA (catalog A30-103A, Bethyl Laboratories Inc., Montgomery, TX) was added in a dilution of 1:2,000. After washing with PBS-t rabbit-anti-goat IgG (catalog A50-100P, Bethyl Laboratories Inc.) was added in a dilution of 1:500. After washing in PBS sections were incubated in a Na-acetate buffer solution and subsequently stained with amino-ethyl-carbazole. After washing with distilled water sections were counterstained with hematoxylin and mounted using Aquamount.

Controls consisted of 3 sections per bird, which were treated as described above, but to which no goat-anti-chicken IgA was added. No staining was observed for any of these control sections.

Stainings for IgA were in the first instance performed on a limited number of samples from both breeds to get a first impression on possible effects of EF on IgA+ cell numbers. From those limited samples, it seemed that there might be an effect of EF on IgA+ cell numbers in broilers, but not in layers. It was then decided to focus more extensively on broilers with regard to IgA+ cells.

### ***ELISA Procedure***

Total NAb titers against KLH were determined by ELISA. Briefly, 96 wells plates were coated with 100  $\mu$ L of 0.1 M carbonate buffer (pH 9.6) containing 4  $\mu$ L/mL of KLH. All washing steps were conducted with tap water containing 0.05% Tween. After washing, plates were incubated for 90 min at RT with serial 4-step double dilutions of serum in PBS containing 0.05% Tween and 0.5% normal horse serum. After washing, plates were incubated for 90 min at RT with goat-anti-chicken IgY<sub>Fc</sub> (catalog A30-104P, Bethyl Laboratories Inc.; dilution 1:40,000) or Goat-anti-chicken IgM (catalog A30-102P, Bethyl Laboratories Inc.; dilution 1:20,000) in PBS containing 0.05% Tween and 0.5% normal horse serum. After washing, plates were incubated with tetramethylbenzidine at RT and after 10 min the reaction was stopped with 1.25% H<sub>2</sub>SO<sub>4</sub>. Extinctions were measured with a Thermo Scientific Multiskan GO microplate spectrophotometer at a wavelength of 450 nm. Differences of NAb titers between treatment groups over time were determined and titers were not related to titers of d 0.

### Statistical Analysis

Relative expression levels of the genes of interest were analyzed for significant differences between treatments and tissue sampling time points using PROC MIXED in SAS 9.2 (SAS Institute Inc., Cary, NC). Normality was checked on the residuals. A natural log-transformation was performed on the data in order to approximate a normal distribution.

Because broilers and layers were kept in 2 different rooms, breed was confounded with room and therefore breeds were analyzed separately. For this reason, differences between broilers and layers will be discussed in a descriptive way. Expression of 28S was not influenced by breed or treatment, making a descriptive comparison regarding gene expression possible between breeds.

The statistical model applied to the data was as follows:

$$Y_{ijk} = \mu + \text{Treat}_i + \text{Time}_j + (\text{Treat} \times \text{Time})_{ij} + \varepsilon_{ijk},$$

where  $Y_{ijk}$  represents the  $ijk$ th relative expression of IL-1 $\beta$ , IL-10, IL-12p40, IFN- $\gamma$ , TGF- $\beta$ , IgA, IgM, IgY, number of IgA+ cells, IgM and IgY antibody titers against KLH, spleen weight, or bursa weight. The mean is represented by  $\mu$ .  $\text{Treat}_i$  represents the fixed class effect of treatment administered to birds ( $i = \text{EF, DF}$ ).  $\text{Time}_j$  represents the fixed class effect of time points on which tissue sampling occurred ( $j = 0, 3, 6, \dots, 42$  for broilers and  $0, 3, 6, \dots, 140$  for layers). Interaction between treatments and time points is represented by the fixed class effect  $(\text{Treat} \times \text{Time})_{ij}$  and is used to determine significant differences between treatments per tissue sampling time point. The random residual term from a normal distribution is represented by  $\varepsilon_{ijk}$ . Effects of treatment on BW were calculated per d.

Results are displayed as means along with the SE. Differences are considered as significant where  $P \leq 0.05$ .

## RESULTS

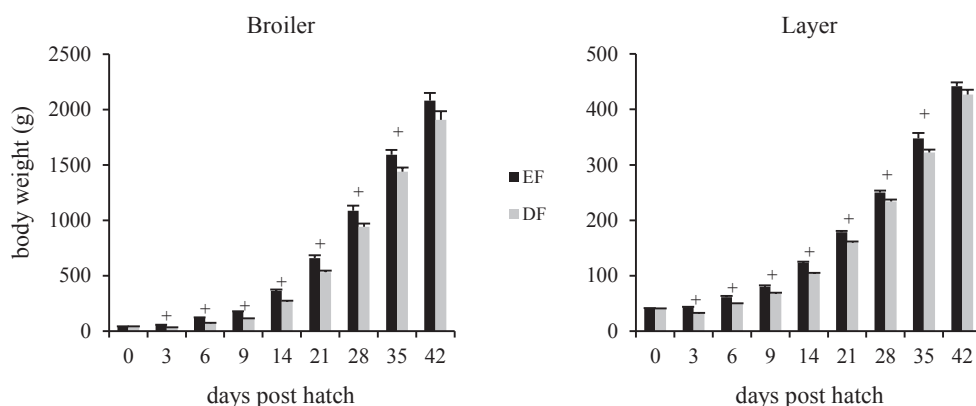
### *BW*

Body weight of EF chicks was higher from d 3 through d 35 in both breeds ( $P < 0.05$ ; Figure 1). On d 42, DF chicks seemed to have caught up on BW because significant differences with EF chicks were no longer present. For layers, similar BW between feeding treatments were found until the end of the experimental period on d 140 (data not shown).

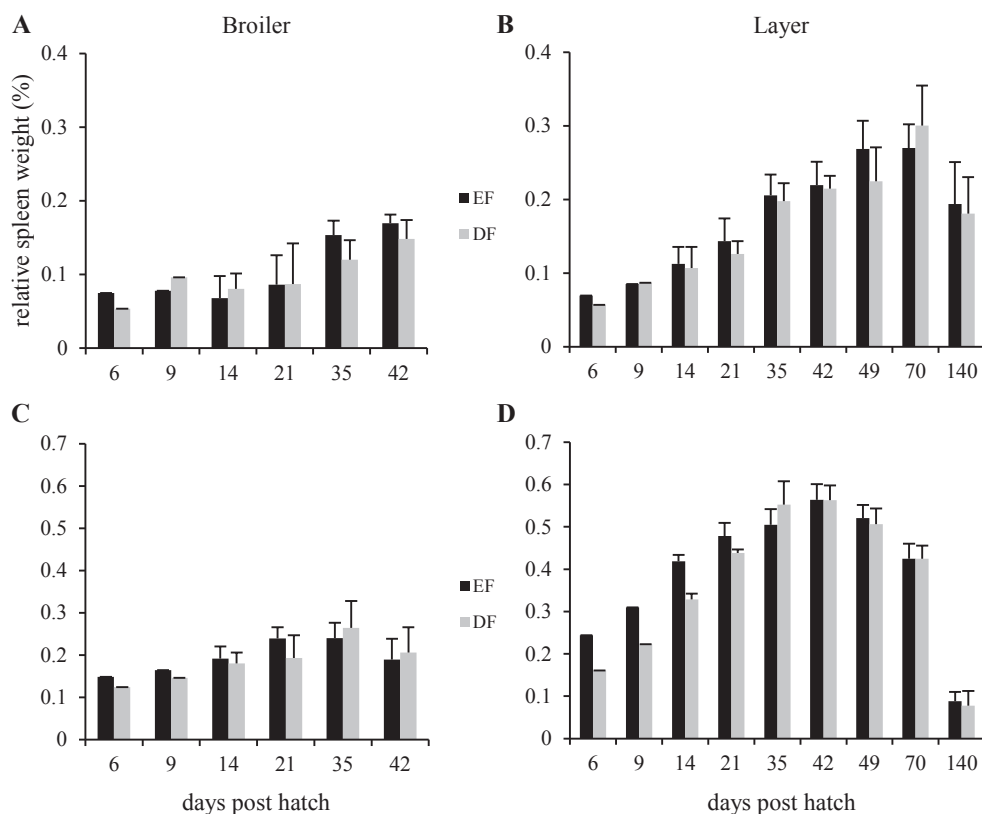
### *Relative Spleen and Bursa Weight*

In broilers, relative spleen and bursa weight were not influenced by EF. Both lymphoid organs gradually increased in weight over time ( $P < 0.01$ ; Figure 2A and 2C).

In layers, relative spleen weight was generally higher in EF chicks ( $0.174\% \pm 0.009$ ) compared with DF chicks ( $0.167\% \pm 0.009$ ,  $P = 0.03$ ). It seems that this effect was mainly caused by generally higher spleen weights in the EF group until d 49 (Figure 2B). Relative bursa weight was not affected by treatment (Figure 2D). Both relative spleen and bursa weight increased in time ( $P < 0.01$ ). Relative spleen weight increased until d 70 and showed a decrease on d 140. After a continuous increase, bursa weight started to decrease from d 49 onward and showed a severe drop on d 140.



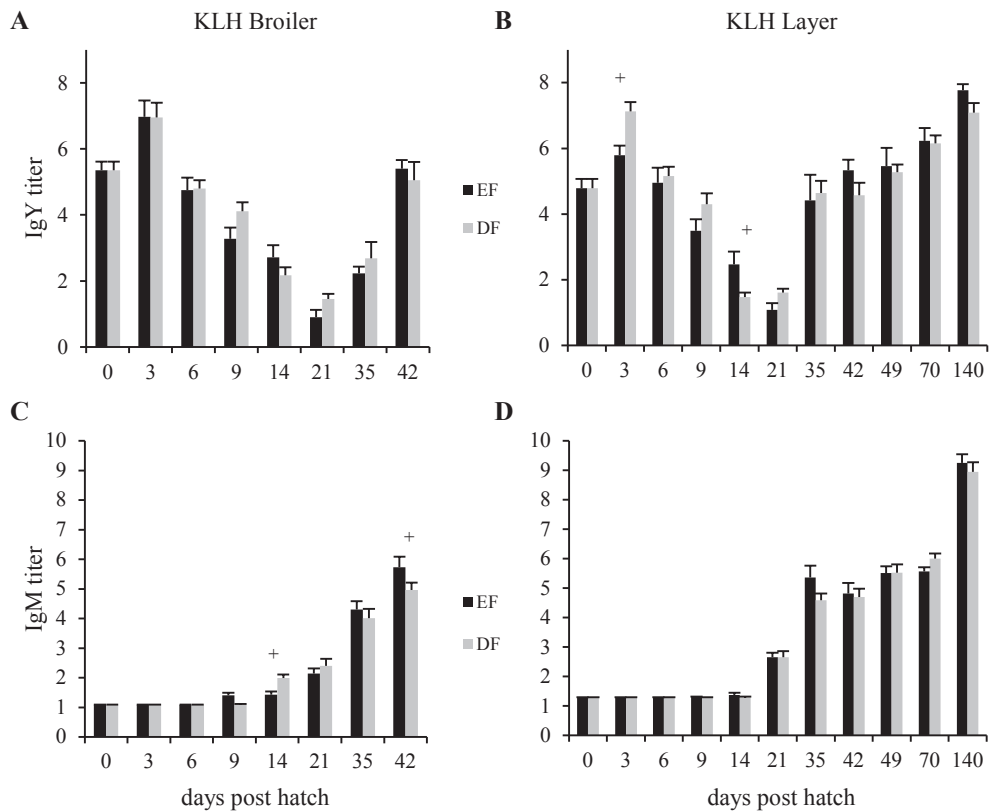
**Figure 1.** Body weight of broilers and layers during the first 42 d post hatch. Treatments were early feeding (EF) and delayed feeding (DF). Data are displayed as means and corresponding SE. Significant differences ( $P < 0.05$ ) between treatments within a time point are indicated with a plus.



**Figure 2.** Relative spleen and bursa weight. Treatments were early feeding (EF) and delayed feeding (DF). A: Relative spleen weight of broilers, B: Relative spleen weight of layers, C: Relative bursa weight of broilers, D: Relative bursa weight of layers. Data are displayed as means and corresponding SE.

### Systemic Natural Immunoglobulin Levels

For both broilers and layers, IgY antibody levels against KLH showed an increase on d 3 p.h., after which levels decreased to a minimal level on d 21 (Figure 3A). Thereafter, an increase in IgY levels was observed on d 35, which was clearly more pronounced in layers (difference of 2 titer units between layers and broilers; Figure 3B), but levels were similar again on d 42. Levels of IgY continued to increase after d 35. In broilers only time affected IgY levels ( $P < 0.01$ ), whereas



**Figure 3.** Titers of IgY and IgM against keyhole limpet hemocyanin (KLH) of broilers (A and C) and layers (B and D). Treatments were early feeding (EF) and delayed feeding (DF). Data are displayed as means and corresponding SE. Significant differences ( $P < 0.05$ ) between treatment groups within one time point are indicated with a plus.

in layers a time  $\times$  treatment interaction ( $P = 0.05$ ) was observed. In layers, IgY levels in DF chicks (titer  $7.1 \pm 0.3$ ) were higher on d 3 compared with EF chicks (titer  $5.8 \pm 0.3$ ,  $P < 0.01$ ), whereas on d 14 DF chicks had lower (titer  $1.5 \pm 0.1$ ) levels of IgY compared with EF chicks (titer  $2.5 \pm 0.4$ ,  $P = 0.05$ ).

Levels of IgM started to increase in EF broiler chicks from d 9 and in DF chicks from d 14 onward. Thereafter, a continuous increase in IgM levels was observed for both treatments (Figure 3C). A time  $\times$  treatment interaction ( $P = 0.01$ ) was observed for broilers caused by higher IgM levels in DF chicks on d 14 (titer 2

$\pm 0.1$ ) compared with EF chicks (titer  $1.4 \pm 0.1$ ,  $P = 0.02$ ), whereas DF chicks showed lower levels on d 42 (titer  $5 \pm 0.2$ ) compared with EF chicks (titer  $5.7 \pm 0.4$ ,  $P < 0.01$ ). For layers, IgM antibody levels against KLH remained low through d 14 p.h. On d 21, IgM levels strongly increased until d 35. After that, IgM levels plateaued until another sharp increase in levels on d 140 (Figure 3D). In layers, IgM levels were only affected by time ( $P < 0.01$ ).

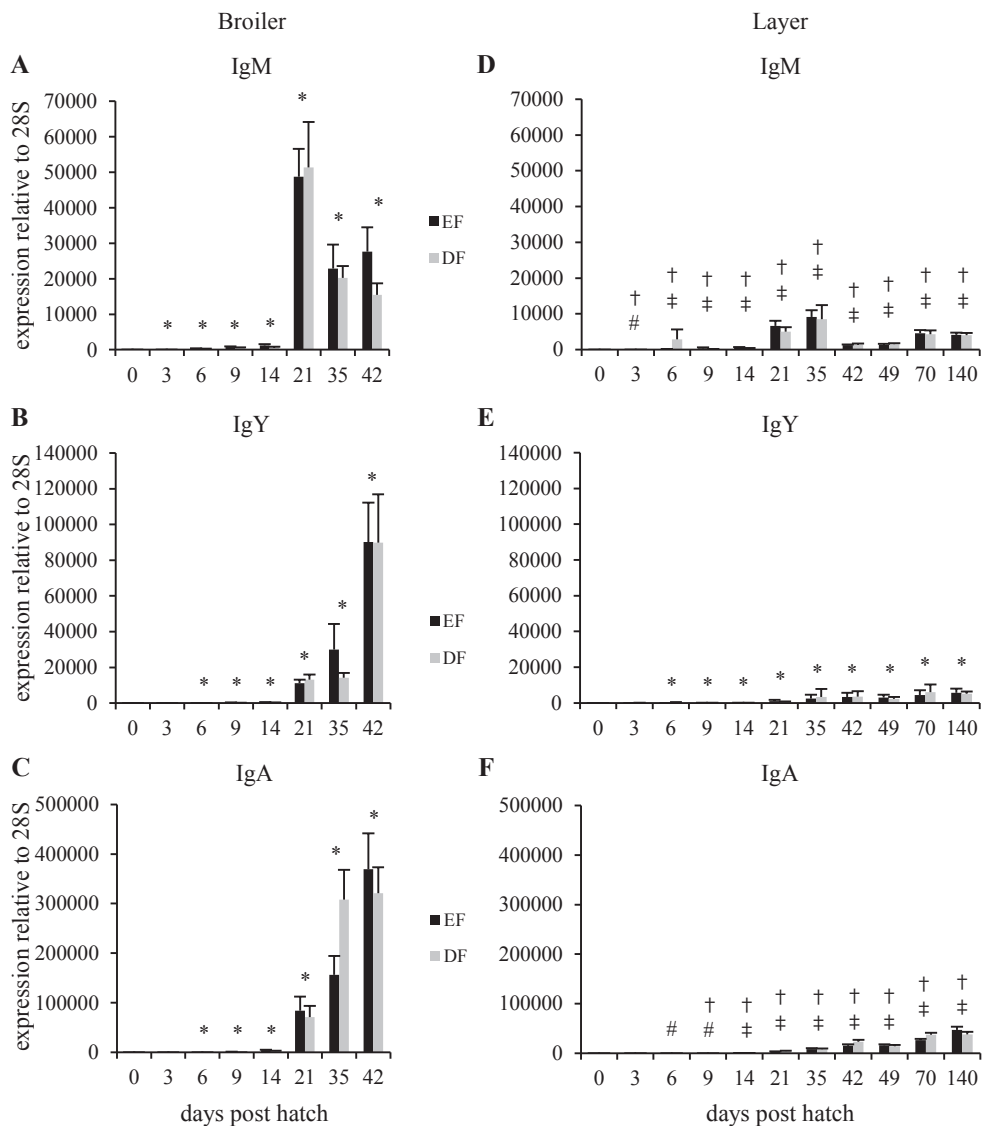
### ***Ileal Immunoglobulin Expression Levels & IgA<sup>+</sup> Cells***

Relative immunoglobulin mRNA expression levels of all measured time points were compared with expression levels of d 0. Immunoglobulin expression levels showed similar kinetics in both breeds, but were remarkably higher in broilers. Expression levels of IgM were up to 16 times higher (d 42), IgY expression levels up to 26 times higher (d 42), and IgA expression levels up to 28 times higher (d35) in broilers.

Relative IgM, IgY, and IgA expression levels strongly increased on d 21 in both breeds (Figure 4A through 4F). The IgM levels in broilers dropped again slightly on d 35 and 42, whereas IgY and IgA showed a continuous increase after d 21. The IgM levels in layers dropped on d 42, after which levels remained lower compared with d 21 and 35 throughout the rest of the experimental period. After the increase on d 21 IgY and IgA levels in layers showed a continuous rise, disrupted by a slight transient drop in levels on d 49.

The IgM expression levels in broilers were influenced by both time ( $P < 0.01$ ) and treatment ( $P = 0.03$ ). Over the whole experimental period, EF chicks had higher IgM expression levels compared with DF chicks and IgM expression levels exceeded those of d 0 from d 3 onward.

For IgM expression levels in layers, a time x treatment interaction ( $P < 0.01$ ) was found. The EF and DF chicks differed within one time point on d 3, on which IgM expression levels were 4 times lower in EF chicks. The IgM expression levels of EF chicks showed a significant drop on d 3 compared with d 0. The IgM expression levels of both EF and DF chicks exceeded those of d 0 from d 6 onward.



**Figure 4.** Real-time quantitative PCR results of immunoglobulin mRNA expression levels in mid-sections of ileum in broiler (A through C) and layer (D through F) chickens. Expression levels are displayed as means and corresponding SE. Treatments were early feeding (EF) and delayed feeding (DF). Significant differences ( $P < 0.05$ ) between treatments within each d and between d 0 and following ds are indicated with a symbol above bars. Symbols indicate the following significant differences: # significant difference between the EF and DF group within a time point; † significant difference from d 0 for the EF group; ‡ significant difference from d 0 for the DF group; \* significant difference from d 0 for the EF and DF group combined as a total (main effect of d).

For IgY expression levels, a time effect was found in both broilers ( $P < 0.01$ ) and layers ( $P < 0.01$ ). In both breeds, overall IgY expression levels exceeded those of d 0 from d 6 onward.

The IgA expression levels in broilers were influenced by time ( $P < 0.01$ ) and treatment ( $P = 0.03$ ). Over the whole experimental period EF chicks had higher IgA expression levels compared with DF chicks. This overall treatment effect was mainly caused by higher IgA levels in the EF group on d 6 through 14. The IgA expression levels exceeded those of d 0 from d 6 onward.

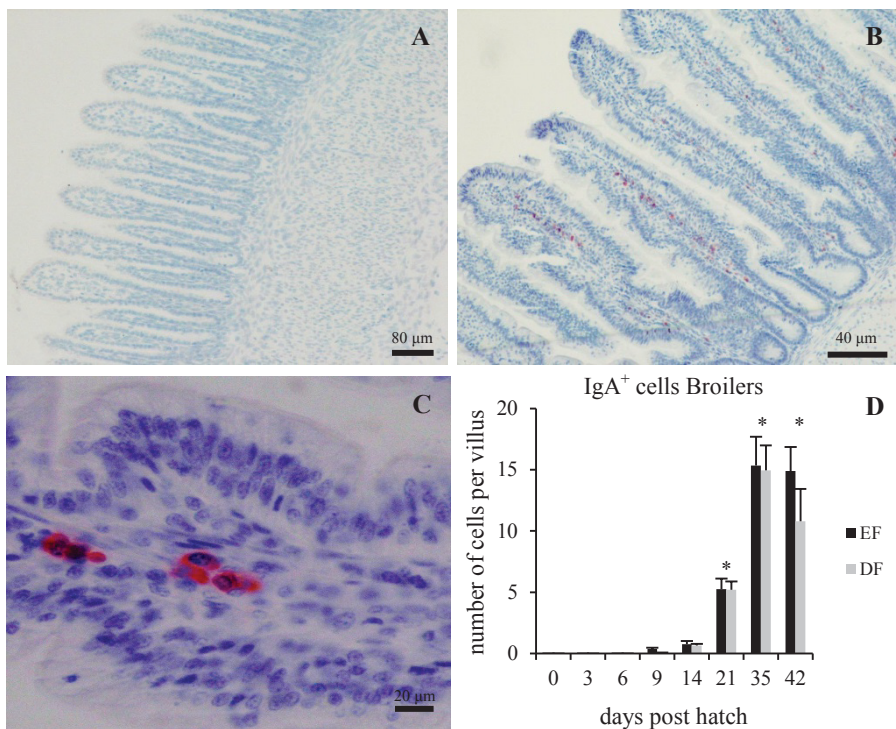
The IgA<sup>+</sup> cells in broilers were visible for the first time on d 9, and like IgA expression levels, showed a dramatic increase on d 21 and continued to increase through d 35 (Figure 5C). The number of IgA<sup>+</sup> cells was influenced by time ( $P < 0.01$ ). Although the first cells were visible on d 9, numbers of IgA<sup>+</sup> cells did not differ from d 0 (Figure 5A) until the dramatic increase in cells on d 21 (Figure 5B). Unlike for IgA expression levels, EF did not have an effect on the number of IgA<sup>+</sup> cells.

For IgA expression in layers, a time x treatment interaction ( $P = 0.01$ ) was found. The EF and DF chicks differed within one time point on d 6 and 9. On d 6, DF chicks showed higher IgA expression levels compared with EF chicks, whereas the opposite was true for d 9. The IgA expression levels of EF chicks exceeded those of d 0 from d 9 onward, whereas IgA expression levels of DF chicks exceeded those of d 0 from d 14 onward.

### ***Ileal Cytokine Expression Levels***

Relative cytokine expression levels of all measured time points were compared to expression levels of d 0. No effect of EF or DF was observed for any of the cytokines in both breeds. Cytokine expression levels differed between breeds regarding their kinetics and were remarkably lower in broilers. Expression levels were up to 17 times (d 9) lower for IL-12p40 in broilers, up to 13 times (d 9) lower for IL-1 $\beta$ , up to 39 times (d 14) lower for IFN- $\gamma$ , up to 49 times (d 14) lower for IL-10, and up to 3 times (d 14) lower for TGF- $\beta$ .

In both broilers and layers, IL-12p40 (Figure 6A and 6F) was present from the beginning and did not show a clear pattern over the experimental period, but was characterized by several ups and downs in expression levels. For both breeds, a



**Figure 5.** Ileal IgA<sup>+</sup> cells in broilers. A: Ileal IgA<sup>+</sup> cells on d 0. B and C: Ileal IgA<sup>+</sup> cells on d 21. D: Number of IgA<sup>+</sup> cells per villus. Treatments were early feeding (EF) and delayed feeding (DF). Data are displayed as means and corresponding SE. Significant differences with d 0 ( $P < 0.05$ ) are indicated with an asterisk.

time effect was observed for IL-12p40 expression levels ( $P < 0.01$ ). With the exception of a transient drop on d 9, broilers maintained basal IL-12p40 expression levels throughout the whole experimental period. Layers showed an elevation of IL-12p40 expression levels on d 14 and 21 after which levels dropped to basal levels and increased again on d 49. This was followed by another transient drop to basal levels on d 70.

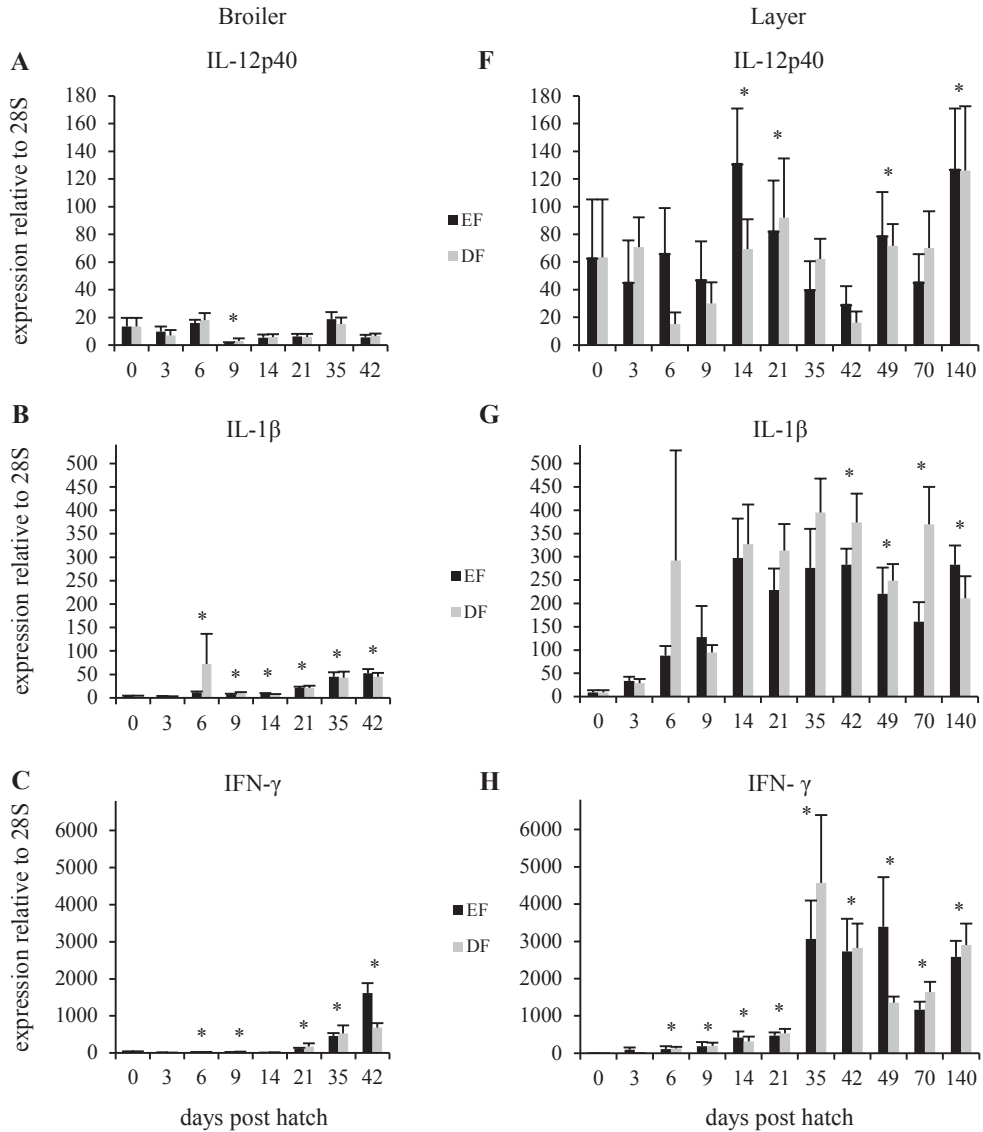
For IL-1 $\beta$  (Figure 6B and 6G), both breeds showed an increase in expression levels on d 6, which was most obvious in the DF group. Thereafter, broilers showed a transient decrease until levels started to increase slowly from d 21 onward. In layers, a transient decrease was observed only for d 9, after which

levels increased sharply on d 14 and remained on a more or less similar level throughout the rest of the experimental period. A time effect on IL-1 $\beta$  expression levels was found for both breeds ( $P < 0.01$ ). In broilers, IL-1 $\beta$  expression levels exceeded those of d 0 from d 6 onward, in layers from d 3 onward.

For IFN- $\gamma$  expression levels, a steady increase from d 21 onward was observed in broilers (Figure 6C). In layers (Figure 6H), a steep increase in IFN- $\gamma$  expression levels was observed on d 35 after which levels seemed to decrease again slightly until d 70. Thereafter, an increase in IFN- $\gamma$  expression levels was observed again on d 140. For both breeds, a time effect was observed for IFN- $\gamma$  expression levels ( $P < 0.01$ ). In broilers, IFN- $\gamma$  expression levels exceeded those of d 0 on d 6 and 9 for the first time after which they dropped again to basal levels on d 14. Another increase was observed from d 21 onward. In layers, IFN- $\gamma$  expression levels exceeded those of d 0 from d 6 onward and remained above basal levels throughout the rest of the experimental period.

Generally speaking, IL-10 (Figure 6D) as well as TGF- $\beta$  (Figure 6E) expression levels showed a steady increase from d 21 onward in broilers. A time effect was found for both cytokines ( $P < 0.01$ ). The IL-10 as well as TGF- $\beta$  expression levels showed a decrease on d 3, after which a return to basal levels was observed with the exception of a transient increase in levels on d 6 for TGF- $\beta$ . A major increase in levels was observed from d 21 onward for TGF- $\beta$ , which was also observed in IL-10 from d 35 onward.

In layers, IL-10 expression levels (Figure 6I) generally showed a steady increase from d 9 onward with a transient drop in levels on d 21. After d 35, IL-10 expression levels decreased again and remained at lower levels for the rest of the experimental period. For TGF- $\beta$  (Figure 6J), a steady increase was observed until d 70 after which expression levels seemed to decrease on d 140. A time effect was observed for both cytokines ( $P < 0.01$ ). The IL-10 expression levels exceeded those of d 0 from d 9 onward, whereas TGF- $\beta$  expression levels exceeded those of d 0 already from d 6 onward.



**Figure 6.** Real-time quantitative PCR results of cytokine mRNA expression levels in mid-sections of ileum in broiler (A through E) and layer (F through J) chickens. Expression levels are displayed as means and corresponding SE. Treatments were early feeding (EF) and delayed feeding (DF). Significant differences ( $P < 0.05$ ) between d 0 and following days are indicated with an asterisk. IFN = interferon; TGF = transforming growth factor. (continued on the next page)

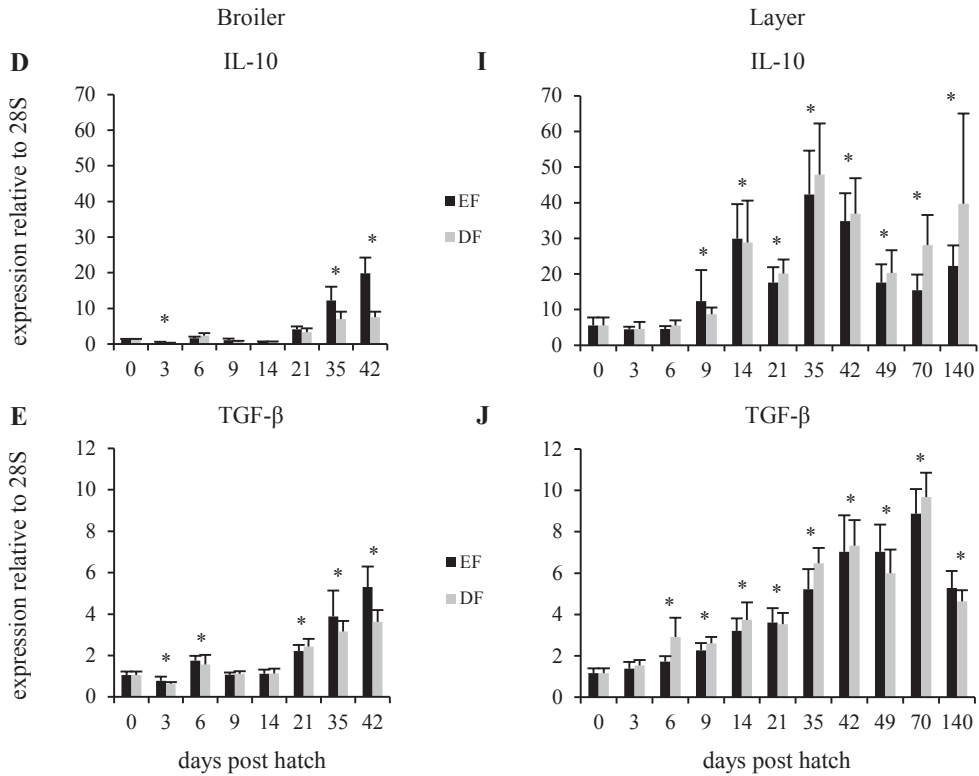


Figure 6. (continued)

## DISCUSSION

This study investigated effects of EF on development of systemic NAb, and ileal immunoglobulin and cytokine expression in broilers and layers during the entire period of immune development. Effects of EF on spleen and bursa weight as well as BW were also taken into account.

According to expectations, EF clearly affected BW in broilers as well as layers. Broilers and layers that had immediate access to feed p.h. showed higher BW between d 3 and d 35 p.h. This result is in accordance with other studies, in which EF broilers showed increased growth compared with those subjected to a

delayed access to feed p.h. or feed restriction in early life (Palo et al., 1995; Noy and Sklan, 1999; Gonzales et al., 2003; Careghi et al., 2005). Early feeding has been shown to lead to an increase in intestinal surface area (Uni et al., 1998; Noy et al., 2001) and an increase in activity of digestive enzymes (Corless and Sell, 1999) and may therefore lead to an improved uptake of nutrients leading to faster growth. On d 42, DF chicks of both breeds seemed to have caught up in weight, and no differences between DF and EF chicks were observed from that day onward.

Immune maturation was expected to be influenced by EF because EF probably stimulates microbial colonization of the intestine and microbial colonization may in turn affect immune development. Multiple studies in humans and other animal species have shown that diet influences microbiota composition (Collins and Gibson, 1999; Penders et al., 2006; Turnbaugh et al., 2009) and that microbiota composition may influence immune development by for example inducing  $T_{reg}$  responses (Geuking et al., 2011) and class switching in B cells (He et al., 2007). In line with these studies, an increase in bacterial numbers can be observed immediately after the first intake of feed in chickens (Shapiro and Sarles, 1949) and a more rapid morphological intestinal development can be observed when food is available in the immediate p.h. period (Uni et al., 1998; Noy et al., 2001). Early feeding studies in broilers seem to be in line with the idea that diet can have some influence on parts of the immune system via influencing the intestinal microbiota composition. Early feeding led to an increased weight of the bursa (Dibner et al., 1998; Bar Shira et al., 2005) as well as a more rapid colonization of the hindgut and bursa with T and B lymphocytes (Bar Shira et al., 2005). Furthermore, more pronounced primary antibody responses were observed in EF chicks (Bar Shira et al., 2005). Lymphoid organs were therefore expected to be heavier in EF chicks as a first indicator of enhanced immune development. Effects of EF on lymphoid organ weights were, however, not as pronounced as in other studies. In fact, EF generally enhanced relative spleen weight in layers only. When analyzing organ weights per time point, however, EF layers did show significantly higher relative bursa weights on d 6 and 9. This was not found for broilers.

Because NAb, which are present without prior exposure to the respective antigen (Avrameas, 1991), form an important part in the first line of defense (Ochsenbein and Zinkernagel, 2000), NAb titers against KLH were also taken account in this study. Natural antibody production in chickens has been shown to

be enhanced by probiotics administered on the d of hatch (Haghighi et al., 2006), indicating that NAb levels may be influenced by microbiota composition and presence of dietary antigens. Early feeding might therefore enhance NAb levels against KLH, but effects of EF were not pronounced and inconsistent in the present study. Both IgM and IgY NAb levels against KLH were, with exception of IgY levels on d 35, similar in both breeds. The fact that IgY in layers is 2 titre units higher at d 35 may indicate that development of systemic IgY levels is faster in layers than in broilers.

Regarding relative immunoglobulin expression levels in the ileum, EF did have some effects in the present study. Early feeding led to generally higher IgM as well as IgA expression levels in broilers throughout the whole experimental period. In contrast to IgA expression levels, numbers of IgA<sup>+</sup> cells in broilers were not affected by EF, which could suggest that in the intestine EF stimulates activity of immune-related genes, but not proliferation of immune cells. It should also be taken into account, however, that gene activation does not necessarily result in production of its protein. In layers, EF led to an earlier onset of IgA expression. Onset of IgA expression occurred 5 d earlier in EF chicks (d 9) compared with DF chicks (d 14). These results could suggest that antigenic stimulation via feed intake contributes to B cell development and isotype-switching as has also been suggested by studies with germ-free animals. Studies with germ-free animals indicate that B cell differentiation and isotype-switching from IgM to IgA depend on intestinal microbiota (Benevise et al., 1971; Moreau et al., 1978; He et al., 2007; Liu and Rhoads, 2013). Intestinal microbiota composition, however, needs to be of a certain complexity as shown in the study of Moreau et al. (1978) with germ-free and gnotobiotic mice. While certain gram-negative strains were found to increase duodenal IgA<sup>+</sup> cells, others did not. When combining several, individually not stimulatory strains, however, an increase in IgA<sup>+</sup> cells was observed. This necessity of a certain complexity in microbiota composition for an isotype-switch to IgA may have been the reason why onset of IgA expression slightly lagged behind DF layer chicks, in which microbial succession may have been delayed due to a delay in feed intake. Future studies should investigate whether EF indeed increases microbial diversity.

Furthermore, EF had only limited effects on ileal immune maturation in the present study. Early feeding did not affect onset of cytokine expression or cytokine

expression levels. Apart from that, the onset of IgM and IgY expression were not affected by EF either, and EF also had no effects on IgY expression levels. These results are in line with a study from Bar Shira et al. (2005), who found that EF had only limited effects on antibody and cytokine expression in the foregut, whereas EF did have effects on antibody and cytokine expression in the hindgut.

It should be realized that the observations in the present study were made in healthy, nonchallenged chicks. Long-term effects of feeding strategy on (mucosal) immune system and metabolism may manifest itself more clearly in stressed or challenged animals.

Of special interest is the clear difference between broilers and layers regarding immunological development in the ileum. Whereas generally speaking broilers in this study showed a decrease in cytokine expression levels between 2 and 3 wk p.h., layers generally showed an increase in cytokine expression levels in the period between 2 and 6 to 7 wk p.h. Upregulation of cytokine expression levels during the 2 to 6 wk p.h. period has been observed earlier in layers (Lammers et al., 2010). It was suggested that this highly dynamic period is necessary for activation of the local adaptive immune system and in particular production of IgA, which is regarded the principal component of mucosal immunity. Lammers et al. (2010) have suggested a role of IL-10, TGF- $\beta$ , and IFN- $\gamma$  in class switching of B cells towards IgA production in chickens and have furthermore indicated that class switching might be, at least partly, T cell-dependent in layers. The downside of T cell-dependent production of IgA is its relatively time-consuming process, which can be a disadvantage in the intestinal environment, which is abundant in commensal bacteria, possible pathogens, as well as antigens derived from feed (Cerutti, 2008). Therefore, a second, T cell-independent pathway exists, which is able to induce IgA class switching in a fast way in the lamina propria (Cerutti, 2008). The IgA produced in a T cell-independent manner consists of unspecific, low affinity IgA, which is able to neutralize antigens by being able to bind a wide range of epitopes and in doing so prevents translocation of commensal bacteria (Fagarasan and Honjo, 2000). The fact that broilers in the present study had 28 times higher relative mRNA expression of IgA than layers without a clear upregulation of any of the above mentioned cytokines, including TGF- $\beta$ , might suggest that intestinal IgA class switching is mainly T cell-independent in broilers. In that case, the complete IgA repertoire in broilers may have characteristics of

NAb and have a polyspecific nature. For future studies, it might be interesting to investigate factors involved in T cell-dependent class switching, such as CD40L, and T cell-independent class switching, such as B cell activating factor BAFF, in both breeds.

A possible reason for a lower expression of cytokines and a higher expression of immunoglobulins might be that energetic costs for antibody responses are lower (Van Eerden et al., 2004; Korver, 2012) compared with acute phase responses characterized by a massive release of cytokines (Husband, 1995). It might therefore be possible that this relatively costly part of the immune system has been sacrificed in broilers by selection for optimal growth and that broilers at the same time make up for this deficit by higher production of relatively cost-efficient immunoglobulins, which can neutralize a wide array of pathogens. Another possibility might be an unintentional selection for high mucosal immunoglobulin levels, which prevent absorption of antigens (Woof and Mestecky, 2005) and therefore prevent the stimulation of immune cells.

There were also similarities observed between broilers and layers. Both breeds behaved in a similar way regarding onset of immunoglobulin expression levels. Immunoglobulin expression levels were close to zero at hatch, but started to increase within the first 2 wk p.h. In both breeds, IgM and IgY expression levels exceeded those of d 0 within the first week. For IgA this was also true for broilers, but not for layers. Layers showed an increase in IgA expression levels above those of d 0 within the second week p.h. Although immunoglobulin expression levels started to increase quite early, a more dramatic increase in all immunoglobulin levels was observed on d 21 in both breeds.

In conclusion, this study shows that EF does not seem to have a clear effect on ileal immune maturation, but it also demonstrates that broilers and layers differ considerably in their immune development on ileal level. Broilers show up to 28 times higher immunoglobulin and up to 49 times lower cytokine expression levels compared with layers. Regarding cytokine expression the 2 breeds also differ in their kinetics. These differences in immunological traits may have been caused by genetic selection for different production traits in the 2 breeds and suggest that these 2 extreme breeds use different immune strategies to remain healthy. Furthermore, the results of the present study could suggest that for the purpose of

dietary immune modulation in order to improve disease resistance, the 2 chicken types require different feed additives with a different mode of action.

### **ACKNOWLEDGEMENTS**

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## REFERENCES

- Avrameas, S. 1991. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol. Tod* 12:154-159.
- Bar-Shira, E., D. Sklan, and A. Friedman. 2003. Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Dev. Comp. Immunol.* 27:147-157.
- Bar Shira, E., D. Sklan, and A. Friedman. 2005. Impaired immune responses in broiler hatchling hindgut following delayed access to feed. *Vet. Immunol. Immunopathol.* 105:33-45.
- Beneviste, J., G. Lepsinats, C. Adam, and J.-C. Salomon. 1971. Immunoglobulins in Intact, Immunized, and Contaminated Axenic Mice: Study of Serum IgA. *J. Immunol.* 107:1647-1655.
- Careghi, C., K. Tona, O. Onagbesan, J. Buyse, E. Decuypere, and V. Bruggeman. 2005. The effects of the spread of hatch and interaction with delayed feed access after hatch on broiler performance until seven ds of age. *Poult. Sci.* 84:1314-1320.
- Cerutti, A. 2008. The regulation of IgA class switching. *Nat. Rev. Immunol.* 8:421-434.
- Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16:228-231.
- Collins, M. D., and G. R. Gibson. 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.* 69:1052s-1057s.
- Corless, A. B., and J. L. Sell. 1999. The effects of delayed access to feed and water on the physical and functional development on the digestive system of young turkeys. *Poult. Sci.* 78:1158-1169.
- De Filippo, C., D. Cavalieri, M. Di Paola, M. Ramazzotti, J. B. Poullet, S. Massart, S. Collini, G. Pieraccini, and P. Lionetti. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S.A.* 107:14691-14696.
- Dibner, J. J., C. D. Knight, M. L. Kitchell, C. A. Atwell, A. C. Downs, and F. J. Ivey. 1998. Early feeding and development of the immune system in neonatal poultry. *J Appl Poult Res* 7:425-436.
- Fagarasan, S., and T. Honjo. 2000. T-independent immune response: new aspects of B cell biology. *Science* 290:89-92.
- Geuking, M. B., J. Cahenzli, M. A. E. Lawson, D. C. K. Ng, E. Slack, S. Hapfelmeier, K. D. McCoy, and A. J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 34:794-806.

- Gonzales, E., N. Kondo, É. S. P. B. Saldanha, M. M. Loddy, C. Careghi, and E. Decuypere. 2003. Performance and physiological parameters of broiler chickens subjected to fasting on the neonatal period. *Poult. Sci.* 82:1250-1256.
- Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, H. Zhou, B. Sanei, J. R. Chambers, and S. Sharif. 2006. Probiotics stimulate production of natural antibodies in chickens. *Clin. Vaccine Immunol.* 13:975-980.
- He, B., W. Xu, P. A. Santini, A. D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D. M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. *Immunity* 26:812-826.
- Husband, A. J. 1995. The immune system and integrated homeostasis. *Immunol. Cell Biol.* 73:377-382.
- Jeurissen, S. H. M., E. M. Janse, G. Koch, and G. F. De Boer. 1989. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* 258:119-124.
- Korver, D. R. 2012. Implications of changing immune function through nutrition in poultry. *Anim. Feed Sci. Technol.* 173:54-64.
- Lammers, A., W. H. Wieland, L. Kruijt, A. Jansma, T. Straetemans, A. Schots, G. den Hartog, and H. K. Parmentier. 2010. Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. *Dev. Comp. Immunol.* 34:1254-1262.
- Liu, Y., and J. Rhoads. 2013. Communication between B-Cells and Microbiota for the Maintenance of Intestinal Homeostasis. *Antibodies* 2:535-553.
- Maslowski, K. M., and C. R. Mackay. 2011. Diet, gut microbiota and immune responses. *Nat. Immunol.* 12:5-9.
- Moreau, M. C., R. Ducluzeau, D. Guy-Grand, and M. C. Muller. 1978. Increase in the Population of Duodenal Immunoglobulin A Plasmocytes in Axenic Mice Associated with Different Living or Dead Bacterial Strains of Intestinal Origin. *Infect. Immun.* 21:532-539.
- Noy, Y., A. Geyra, and D. Sklan. 2001. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poult. Sci.* 80:912-919.
- Noy, Y., and D. Sklan. 1999. Different Types of Early Feeding and Performance in Chicks and Poults. *J Appl Poult Res* 8:16-24.
- Ochsenbein, A. F., and R. M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunol. Tod* 21:624-630.

- Palo, P. E., J. L. Sell, F. J. Piquer, M. F. Soto-Salanova, and L. Vilaseca. 1995. Effect of early nutrient restriction on broiler chickens. 1. Performance and development of the gastrointestinal tract. *Poult. Sci.* 74:88-101.
- Penders, J., C. Thijs, C. Vink, F. F. Stelma, B. Snijders, I. Kummeling, P. A. Van den Brandt, and E. E. Stobberingh. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 118:511-521.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29.
- Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9:313-323.
- Shapiro, S. K., and W. B. Sarles. 1949. Microorganisms in the intestinal tract of normal chickens. *J. Bacteriol.* 58:531-544.
- Turnbaugh, P. J., V. K. Ridaura, J. J. Faith, F. E. Rey, R. Knight, and J. I. Gordon. 2009. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 1:1-10.
- Uni, Z., S. Ganot, and D. Sklan. 1998. Posthatch development of mucosal function in the broiler small intestine. *Poult. Sci.* 77:75-82.
- Van Eerden, E., H. Van Den Brand, H. K. Parmentier, M. C. M. De Jong, and B. Kemp. 2004. Phenotypic selection for residual feed intake and its effect on humoral immune responses in growing layer hens. *Poult. Sci.* 83:1602-1609.
- Wen, L., R. E. Ley, P. Y. Volchkov, P. B. Stranges, L. Avanesyan, A. C. Stonebraker, C. Hu, F. S. Wong, G. L. Szot, J. A. Bluestone, J. I. Gordon, and A. V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109-1113.
- Woof, J. M., and J. Mestecky. 2005. Mucosal immunoglobulins. *Immunol. Rev.* 206:64-82.



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## **CHAPTER 3**

**Early feeding and early life housing conditions influence the response towards a noninfectious lung challenge in broilers**

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## ABSTRACT

Early life conditions such as feed and water availability immediately post hatch (**p.h.**) and housing conditions may influence immune development and therefore immune reactivity later in life. The current study addressed the consequences of a combination of these 2 early life conditions for immune reactivity, i.e., the specific antibody response towards a non-infectious lung challenge. Broiler chicks received feed and water either immediately p.h. or with a 72 h delay and were either reared in a floor or a cage system. At 4 weeks of age chicks received either an intratracheally administered *Escherichia coli* lipopolysaccharide (**LPS**) / Human Serum Albumin (**HuSA**) or a placebo, and antibody titers were measured up to day 14 after administration of the challenge. Chicks housed on the floor and which had a delayed access to feed p.h. showed the highest antibody titers against HuSA. These chicks also showed the strongest sickness response and poorest performance in response to the challenge, indicating that chicks with delayed access to feed might be more sensitive for an environment with higher antigenic pressure. In conclusion, results from the present study show that early life feeding strategy and housing conditions influence a chick's response to an immune challenge later in life. These 2 early life factors should therefore be taken into account when striving for a balance between disease resistance and performance in poultry.

**Key words:** chicken, housing conditions, early feeding, immune response

## INTRODUCTION

Early life conditions may have long-lasting effects on an individual's immune system. One of these early life conditions is post hatch (**p.h.**) access to feed. In poultry production, access to feed is commonly delayed by up to 72 h p.h., which may lead to suboptimal gut and immune development, manifesting in delayed mucosal and villus development (Uni et al., 1998; Lamot et al., 2014) and lower antibody responses after immunization in the first 2 weeks of life (Bar Shira et al., 2005). It has furthermore been suggested that chicks that experienced a delayed access to feed are less well prepared to handle environmental and disease challenges (Dibner et al., 1998). Delayed access to feed p.h., in combination with disinfection procedures at hatcheries, possibly hampers the intestinal microbial colonization process in chicks. Adequate microbial colonization is, however, important for a well-functioning immune system. For example, intestinal microbiota seem to be involved in the direction of class switching in B cells (He et al., 2007) and it has been shown that the mucosal antibody repertoire develops in response to intestinal microbiota (Hapfelmeier et al., 2010). Furthermore, microbial colonization seems to be important for induction of regulatory T cells and T cell homeostasis in the gut (Mazmanian et al., 2005; Hall et al., 2008; Geuking et al., 2011). The interactions between microbiota and the immune system, as well as the fact that intestinal bacterial numbers dramatically increase upon first intake of feed (Shapiro and Sarles, 1949), illustrate the importance of early access to feed p.h. for immune development and immune reactivity later in life.

Apart from access to feed, another factor which may have long-term effects on immune reactivity is early life housing conditions. It has been shown that dust, endotoxin, and bacteria levels are higher in floor systems compared to cage systems (Ellen et al., 2000; Just et al., 2011; Le Bouquin et al., 2013) and differences in antigenic pressure in the 2 housing systems may influence immune reactivity as was shown by a study of Moe et al. (2010). In this study, laying hens reared in a floor system showed higher antibody titers after immunization with sheep red blood cells compared with laying hens reared in a cage system. Housing conditions after the rearing period did not however influence antibody titers, indicating that early life housing conditions may influence immune responses in the long run.

Taken together, both access to feed p.h. and early life housing conditions seem to affect immune responses in chickens later in life, but it remains unclear whether these 2 early life conditions interact in their effects on immune reactivity. The present study therefore investigated immune reactivity in early and delayed fed chickens reared in a floor or cage system.

## MATERIALS AND METHODS

### *Chickens and Housing*

This study was conducted with 128 broiler hens (Ross 308). Fertilized eggs were obtained from a commercial hatchery (Lagerwey Hatchery, Lunteren, The Netherlands) and were incubated at our animal facility under standard conditions. From embryonic day 19 onwards, the eggs were checked regularly for signs of hatching. Every 3 h dry chicks were removed from the incubator and randomly assigned to one of 2 feeding strategies: early feeding (**EF**) or delayed feeding (**DF**). EF chicks received feed and water immediately p.h., DF chicks were given no feed or water for the first 72 h. From 72 h p.h. on, all chicks had ad libitum access to feed and water. Feed consisted of a standard commercial diet containing a coccidiostat (salinomycin). For the first 72 h p.h., all chicks were housed in floor pens on rubber mats in order to prevent ingestion of wood shavings in DF chicks. After 72 h, all EF and DF chicks were housed in groups of 4 in either cages (**C**) of 0.57 x 1.77 m, or in floor pens (**F**) of the same size containing wood shavings, in a 2x2 arrangement, with a regimen of 16L:8D. At 2 wk of age, all chicks received an obligatory Newcastle disease vaccination.

On d 28 p.h., half of the chicks of each feeding strategy and housing conditions received an immunological challenge (**CH**) consisting of intra-tracheally administered LPS (2.5 mg/kg) (from *E. coli* 055:B5, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, catalog L2880) in combination with HuSA (0.5 mg/kg) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, catalog A3782). A combination of LPS/HuSA was used because LPS is known to have an adjuvant effect when administered intra-tracheally in combination with a model antigen, in this case HuSA. Furthermore LPS elicits an innate response, resulting in cachectic

symptoms such as somnolence, and reduced feeding motivation and growth. The other half of the chicks received phosphate buffered saline as a placebo (**PL**) in order to check whether the administered challenge was effective and whether challenged birds responded to the model antigen HuSA. This resulted in 8 treatment groups: 1. early fed cage-housed challenged (**EF-C-CH**), 2. early fed floor-housed challenged (**EF-F-CH**), 3. delayed fed cage-housed challenged (**DF-C-CH**), 4. delayed fed floor-housed challenged (**DF-F-CH**), 5. early fed cage-housed placebo-treated (**EF-C-PL**), 6. early fed floor-housed placebo-treated (**EF-F-PL**), 7. delayed fed cage-housed placebo-treated (**DF-C-PL**), 8. delayed fed floor-housed placebo-treated (**DF-F-PL**). A pen was considered the experimental unit and there were 4 replicates per treatment combination.

This study was approved by the Animal Welfare Committee of Wageningen University and Research Centre in accordance with Dutch laws and regulations on the execution of animal experiments.

### *Measurements and Sampling*

Bodyweight and feed intake were recorded once a week at the same time of day. Additionally growth and feed intake per day was recorded on d -1, 0, 1, and 2 relative to the challenge.

Blood was collected from the wing vein on d -1, 3, 7, and 14 relative to the challenge for determination of antibody titers against LPS and HuSA, and natural antibody (**NAb**) titers against keyhole limpet hemocyanin (**KLH**). Antibody titers were determined by ELISA, as described previously (Simon et al., 2014).

Behavioral measurements in CH birds on the day of the challenge consisted of feeding motivation as part of the sickness response (Johnson, 1998) together with general behavioral observations. Feeding motivation was recorded 24 h before, hourly during the first 7 h after the challenge, and at 48 h after the challenge. Feeding motivation was measured as the latency to approach a mealworm with a maximum of 30 s test time. To avoid food neophobia, birds were exposed to mealworms once a day in the week before the challenge (habituation). General behavior was recorded by means of instantaneous scan sampling every 20 min during the first 7 h after the challenge. Behaviors were assigned to one of 3 categories: resting with closed eyes, resting alert, and active. Resting with closed

eyes included sitting or lying with closed eyes. Resting alert included sitting or lying with open eyes. Active included all other observed behaviors, i.e., drinking, eating, picking on the floor, grooming, (sham) dust bathing, walking, and standing. As broilers at 4 weeks mainly lie or sit due to their weight, standing was regarded an active behavior in the present study. Hourly means per pen were calculated for analysis.

### ***Statistical Analysis***

Data were analyzed using PROC MIXED in SAS 9.2 (SAS Institute Inc., Cary, NC). Normality was checked on the residuals. A natural log transformation was performed on data of antibody titers in order to approximate a normal distribution.

Data on BW and feed intake throughout the whole experimental period, as well as data on NAb titers against KLH, were analyzed per time point. Feeding strategy (EF, DF), housing conditions (cage, floor), and their 2-way interactions were assessed for fixed effects on BW and feed intake before d 28. The administered challenge (CH, PL) was included as a fixed effect in the model after d 28 (day of challenge). One pen was eliminated for feed intake measurements in week 4, because the feeding trough had been knocked over and a large amount of feed had been spilled and could not be retrieved.

Since challenged birds showed higher IgM ( $P = 0.0137$ ) and IgY ( $P = 0.0169$ ) titers against the model antigen HuSA than placebo-treated birds, the challenge was considered successful. Analysis on specific antibody titers against LPS and HuSA, as well as growth, feed intake, feeding motivation and behavior was therefore performed on data for the challenged birds only. For these data, a repeated measures analysis was performed using PROC MIXED. Feeding strategy (EF, DF), housing conditions (cage, floor), and time (days or hours, respectively, relative to administration of the challenge) were assessed for fixed effects on antibody titers against LPS and HuSA, as well as on growth, feed intake, feeding motivation, and behavior in response to the challenge. For feeding motivation, birds which always showed the maximal latency, i.e., never approached the worm during habituation, were excluded from the analysis ( $n = 13$  for EF-C, DF-C, and EF-F;  $n = 16$  for DF-F).

Where not significant, higher order interaction terms were eliminated from the model. All fixed effects were assessed at pen level (experimental unit) by including pen as a random factor in the statistical model. For behavioral data, statistical analyses were performed on pen means. Results are displayed as means and corresponding SE. Differences are considered significant where  $P \leq 0.05$ .

## RESULTS

### BW & Feed Intake

Housing conditions did not influence BW (Table 1A) or feed intake (Table 1B). Feeding strategy however influenced both BW and feed intake throughout the whole experimental period.

From d 3 onward, EF birds showed higher BW than DF birds ( $P < 0.001$  for all days) throughout the whole experimental period, beginning at an average 24.8 g

**Table 1.** Body weight and feed intake per bird throughout the experimental period.

		Treatment Groups			
	d post hatch	EF-C-CH	EF-F-CH	DF-C-CH	DF-F-CH
A. BW (g)	0	44.5 ± 0.8	43.3 ± 0.6	44.4 ± 0.6	45.6 ± 1.1
	3	63.8 ± 1.5 <sup>a</sup>	65.1 ± 1.5 <sup>a</sup>	39.1 ± 0.5 <sup>b</sup>	39.2 ± 0.9 <sup>b</sup>
	7	132.3 ± 3.3 <sup>a</sup>	140.1 ± 4.4 <sup>a</sup>	90.9 ± 2.0 <sup>b</sup>	87.5 ± 2.6 <sup>b</sup>
	14	390.3 ± 12.8 <sup>a</sup>	434.1 ± 11.3 <sup>a</sup>	309.3 ± 6.3 <sup>b</sup>	292.6 ± 7.6 <sup>b</sup>
	21	856.4 ± 21.4 <sup>a</sup>	910.0 ± 18.9 <sup>a</sup>	715.1 ± 15.5 <sup>b</sup>	682.3 ± 15.2 <sup>b</sup>
	28	1479.2 ± 31.6 <sup>a</sup>	1502.1 ± 33.0 <sup>a</sup>	1282.1 ± 20.4 <sup>b</sup>	1220.2 ± 25.6 <sup>b</sup>
	35	2144.4 ± 43.9 <sup>a</sup>	2157.8 ± 49.1 <sup>a</sup>	1937.7 ± 30.0 <sup>b</sup>	1846.0 ± 38.7 <sup>b</sup>
	42	2900.0 ± 43.4 <sup>a</sup>	2918.4 ± 56.3 <sup>a</sup>	2620.9 ± 56.8 <sup>b</sup>	2600.3 ± 52.2 <sup>b</sup>
	wk post hatch				
B. daily feed intake (g)	1	24.0 ± 1.8 <sup>a</sup>	21.7 ± 0.6 <sup>a</sup>	16.3 ± 1.7 <sup>b</sup>	13.4 ± 0.7 <sup>b</sup>
	2	50.4 ± 1.3 <sup>a</sup>	50.8 ± 1.5 <sup>a</sup>	39.8 ± 1.2 <sup>b</sup>	35.0 ± 1.1 <sup>b</sup>
	3	89.0 ± 1.7 <sup>a</sup>	112.8 ± 22.8 <sup>a</sup>	72.0 ± 1.8 <sup>b</sup>	72.3 ± 3.3 <sup>b</sup>
	4	126.9 ± 2.9 <sup>a</sup>	117.6 ± 2.4 <sup>a</sup>	131.0 ± 17.2 <sup>b</sup>	105.8 ± 5.0 <sup>b</sup>
	5	149.6 ± 3.5 <sup>a</sup>	141.5 ± 5.1 <sup>a</sup>	133.9 ± 2.4 <sup>b</sup>	125.8 ± 7.8 <sup>b</sup>
	6	205.9 ± 4.1 <sup>a</sup>	199.8 ± 5.2 <sup>a</sup>	180.6 ± 9.9 <sup>b</sup>	185.5 ± 5.1 <sup>b</sup>

Treatments were combinations of early life feeding strategy (EF = early feeding, DF = delayed feeding), early life housing conditions (F = floor-housing, C = cage-housing) and administration of an intra-tracheally administered challenge (CH = LPS/HuSA, PL = placebo). Data are displayed as means and corresponding SE. Means within a row lacking a common superscript differ significantly ( $P \leq 0.05$ ). (continued on the next page)

Table 1. (continued)

		Treatment Groups			
	d post hatch	EF-C-PL	EF-F-PL	DF-C-PL	DF-F-PL
A. BW (g)	0	42.4 ± 0.8	44.6 ± 0.7	43.8 ± 0.8	44.9 ± 0.5
	3	62.4 ± 1.4 <sup>a</sup>	63.7 ± 1.7 <sup>a</sup>	38.2 ± 0.5 <sup>b</sup>	39.1 ± 0.5 <sup>b</sup>
	7	138.5 ± 3.6 <sup>a</sup>	137.7 ± 3.1 <sup>a</sup>	92.6 ± 1.8 <sup>b</sup>	88.5 ± 1.6 <sup>b</sup>
	14	429.3 ± 10.5 <sup>a</sup>	414.4 ± 12.7 <sup>a</sup>	308.3 ± 9.2 <sup>b</sup>	299.9 ± 6.9 <sup>b</sup>
	21	912.5 ± 17.4 <sup>a</sup>	882.6 ± 24.5 <sup>a</sup>	718.3 ± 18.9 <sup>b</sup>	707.5 ± 14.9 <sup>b</sup>
	28	1543.1 ± 30.6 <sup>a</sup>	1482.1 ± 36.5 <sup>a</sup>	1286.6 ± 45.0 <sup>b</sup>	1286.4 ± 29.5 <sup>b</sup>
	35	2222.3 ± 47.6 <sup>a</sup>	2140.6 ± 44.9 <sup>a</sup>	2003.7 ± 37.7 <sup>b</sup>	1932.6 ± 40.0 <sup>b</sup>
	42	2961.2 ± 71.3 <sup>a</sup>	2902.6 ± 58.1 <sup>a</sup>	2722.9 ± 60.1 <sup>b</sup>	2654.4 ± 58.0 <sup>b</sup>
wk post hatch					
B. daily feed intake (g)	1	24.1 ± 2.2 <sup>a</sup>	21.3 ± 0.2 <sup>a</sup>	15.9 ± 1.0 <sup>b</sup>	12.9 ± 0.6 <sup>b</sup>
	2	49.3 ± 3.2 <sup>a</sup>	48.9 ± 0.4 <sup>a</sup>	40.1 ± 1.4 <sup>b</sup>	36.8 ± 1.2 <sup>b</sup>
	3	91.0 ± 2.3 <sup>a</sup>	133.5 ± 26.0 <sup>a</sup>	77.6 ± 1.9 <sup>b</sup>	104.0 ± 30.4 <sup>b</sup>
	4	134.2 ± 6.3 <sup>a</sup>	124.0 ± 0.7 <sup>a</sup>	113.2 ± 7.1 <sup>b</sup>	114.3 ± 2.9 <sup>b</sup>
	5	142.1 ± 7.4 <sup>a</sup>	142.8 ± 0.5 <sup>a</sup>	139.8 ± 4.2 <sup>b</sup>	135.6 ± 3.2 <sup>b</sup>
	6	200.0 ± 2.1 <sup>a</sup>	197.2 ± 6.7 <sup>a</sup>	191.1 ± 5.9 <sup>b</sup>	185.4 ± 6.3 <sup>b</sup>

higher BW in EF birds on d 3 and ending at an average 271.6 g higher BW in EF birds on d 42.

Mean feed intake per chick per day was higher in EF birds compared to DF birds throughout the whole experimental period ( $P \leq 0.04$  for all time points). Feed intake per day in EF birds was on average between 8.2 g and 25.1 g higher compared with DF birds.

### Natural Antibody Titers

IgM NAb titers against KLH (Table 2A) were influenced by housing conditions, but not feeding strategy or the administered LPS/HuSA challenge. On the day before administration of the LPS/HuSA challenge, IgM titers against KLH were higher in F birds ( $4.0 \pm 0.1$ ) compared with C birds ( $3.6 \pm 0.1$ ) ( $P = 0.041$ ). On d 7 ( $P = 0.036$ ) and d 14 ( $P = 0.001$ ) after administration of the LPS/HuSA challenge IgM titers against KLH were higher in C birds ( $5.7 \pm 0.1$  and  $6.5 \pm 0.2$ , respectively) compared with F birds ( $5.2 \pm 0.1$  and  $5.9 \pm 0.1$ , respectively).

IgY NAb titers against KLH (Table 2A) were not influenced by feeding strategy, housing conditions, or LPS/HuSA challenge.

Table 2. Antibody titers against KLH and LPS in the days following an i.t. LPS/HuSA challenge.

		d after challenge	Treatment Groups							
			EF-C-CH	EF-F-CH	DF-C-CH	DF-F-CH	EF-C-PL	EF-F-PL	DF-C-PL	DF-F-PL
A. KLH IgM	-1 <sup>#</sup>	-1	3.6 ± 0.2	4.1 ± 0.3	3.6 ± 0.2	4.1 ± 0.2	3.7 ± 0.2	4.0 ± 0.4	3.6 ± 0.3	4.1 ± 0.2
		3	4.1 ± 0.3	4.7 ± 0.3	4.2 ± 0.3	4.4 ± 0.2	4.2 ± 0.3	4.5 ± 0.3	3.8 ± 0.3	4.2 ± 0.3
		7 <sup>#</sup>	5.5 ± 0.2	5.5 ± 0.2	5.7 ± 0.2	5.0 ± 0.3	5.8 ± 0.3	5.5 ± 0.2	5.8 ± 0.2	4.8 ± 0.2
	KLH IgY	14	6.6 ± 0.3	5.7 ± 0.2	6.3 ± 0.2	6.2 ± 0.2	6.6 ± 0.3	5.8 ± 0.2	6.6 ± 0.4	6.0 ± 0.2
		-1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.3 ± 0.2	1.3 ± 0.3	1.0 ± 0.1	1.2 ± 0.3
		3	1.2 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.2	1.2 ± 0.2	1.6 ± 0.3
		7	1.9 ± 0.1	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.1 ± 0.2	2.4 ± 0.1	2.4 ± 0.2
B. LPS IgM	-1	-1	3.0 ± 0.2	3.4 ± 0.3	3.1 ± 0.3	2.9 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	3.6 ± 0.4	3.9 ± 0.4
		3	2.1 ± 0.1	2.3 ± 0.2	2.2 ± 0.1	2.3 ± 0.1				
		7	2.6 ± 0.2	2.8 ± 0.3	2.6 ± 0.2	2.5 ± 0.1				
	LPS IgY	14	2.5 ± 0.2	2.7 ± 0.3	2.5 ± 0.2	2.8 ± 0.2				
		-1	3.5 ± 0.2	3.8 ± 0.3	3.6 ± 0.3	3.3 ± 0.1				
		3	1.0 ± 0.2	1.3 ± 0.2	0.8 ± 0.1	1.2 ± 0.2				
		7	1.5 ± 0.2	1.9 ± 0.4	1.2 ± 0.1	1.2 ± 0.2				
		14	1.5 ± 0.2	2.4 ± 0.4	1.8 ± 0.2	2.1 ± 0.3				
			2.1 ± 0.2	2.9 ± 0.3	2.5 ± 0.2	2.4 ± 0.4				

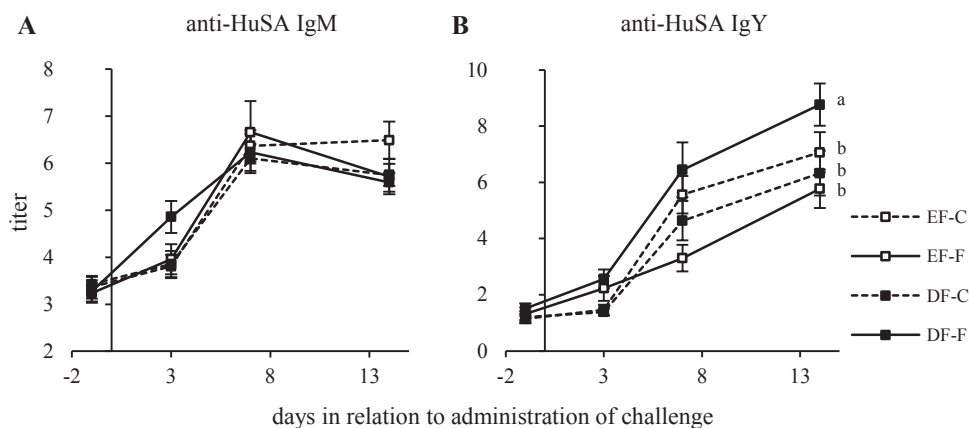
Treatments were combinations of early life feeding strategy (EF = early feeding, DF = delayed feeding), early life housing conditions (F = floor-housing, C = cage-housing) and administration of an i.t. challenge (CH = LPS/HuSA, PL = placebo). Data are displayed as means and corresponding SE. # at time points indicate a significant difference ( $P \leq 0.05$ ) between F and C birds within the respective time point.

### Specific Antibody Titers

IgM titers against HuSA (Figure 1A) were not influenced by feeding strategy or housing conditions.

IgY titers against HuSA (Figure 1B) were influenced by a combination of housing conditions and feeding strategy ( $P = 0.04$ ). On average DF-F birds showed the highest IgY titers against HuSA ( $4.7 \pm 0.5$ ) and differed from EF-C birds ( $3.8 \pm 0.4$ ) ( $P = 0.035$ ), DF-C birds ( $3.3 \pm 0.4$ ) ( $P = 0.011$ ), and EF-F birds ( $3.0 \pm 0.3$ ) ( $P = 0.025$ ). Furthermore, IgY titers against HuSA were influenced by a combination of time and housing conditions ( $P = 0.014$ ). On d 3 after administration of the challenge F birds showed higher IgY titers against HuSA ( $2.4 \pm 0.3$ ) compared with C birds ( $1.4 \pm 0.1$ ) ( $P = 0.002$ ).

Neither IgM nor IgY titers against LPS (Table 2B) were influenced by feeding strategy or housing conditions.

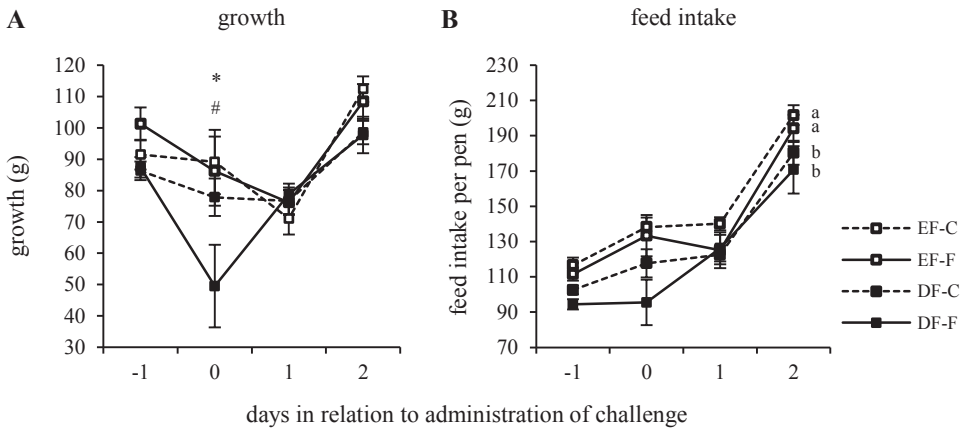


**Figure 1.** Specific antibody titers against HuSA of LPSA/HuSA challenged birds. A: anti-HuSA IgM, B: anti-HuSA IgY. Treatments were combinations of early life feeding strategy (EF = early feeding, DF = delayed feeding) and early life housing conditions (F = floor-housing, C = cage-housing). Data are displayed as means and corresponding SE. # indicates significant differences ( $P \leq 0.01$ ) between F birds and C birds within a time point. Treatment combinations lacking a common superscript differ significantly ( $P \leq 0.05$ ).

**Growth & Feed Intake in Response to LPS/HuSA Challenge**

Growth in response to the LPS/HuSA challenge (Figure 2A) was influenced by a combination of time and feeding strategy ( $P = 0.022$ ) as well as a combination of time and housing conditions ( $P = 0.031$ ). On the day of the challenge, EF birds showed a higher growth ( $87.8 \pm 7.4$  g) compared with DF birds ( $63.2 \pm 7.7$  g) ( $P = 0.001$ ). Furthermore C birds showed a higher growth ( $83.7 \pm 6.0$  g) compared with F birds ( $66.6 \pm 9.2$  g) ( $P = 0.031$ ) on this day. These effects were likely caused by the pronounced growth check in DF-F birds.

Feed intake per day in response to the LPS/HuSA challenge (Figure 2B) was higher in EF birds ( $145.1 \pm 6.1$  g) compared with DF birds ( $126.2 \pm 6.1$  g) ( $P = 0.005$ ). Housing conditions did not influence feed intake in response to the challenge.



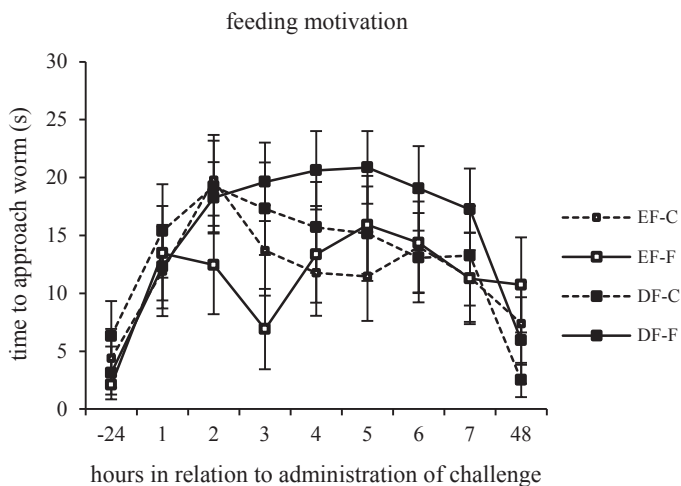
**Figure 2.** Growth and feed intake of LPS/HuSA challenged birds in response to the challenge. A: growth per bird per day, B: feed intake per bird per day. Treatments were combinations of early life feeding strategy (EF = early feeding, DF = delayed feeding) and early life housing conditions (F = floor-housing, C = cage-housing). Data are displayed as means and corresponding SE. \* = significant difference ( $P \leq 0.05$ ) between EF and DF birds within a time point. # = significant difference ( $P \leq 0.05$ ) between F and C birds within a time point. Treatment combinations lacking a common superscript differ significantly ( $P \leq 0.01$ ) in feed intake.

### ***Feeding Motivation in Response to LPS/HuSA Challenge***

Feeding motivation after administration of the LPS/HuSA challenge (Figure 3) was not influenced by feeding strategy or housing conditions. Feeding motivation was influenced by time and was lowest 2 h after administration of the challenge, when birds showed an average latency of  $17.6 \pm 1.8$  s to approach the offered worm. Numerically, feeding motivation was lowest in DF-F birds during the first 7 h after administration of the challenge.

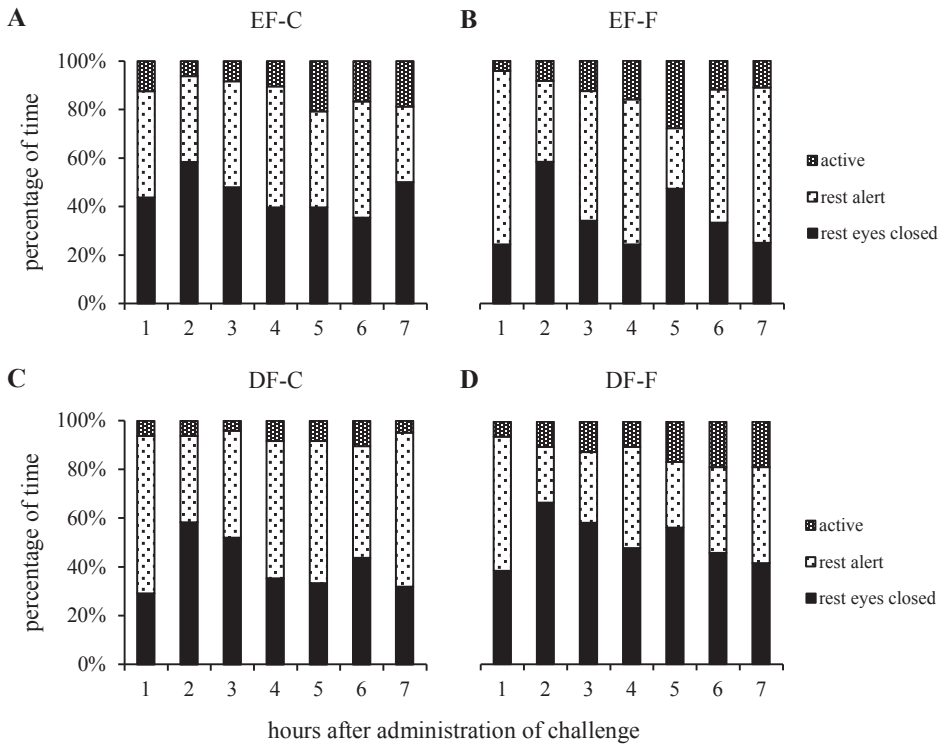
### ***Behavior after LPS/HuSA Challenge***

The behavior of birds in response to the administered LPS/HuSA challenge (Figure 4) was influenced by time. Two h after administration of the challenge, the majority of time ( $60.4 \pm 0.05$  %) was spent resting with closed eyes ( $P < 0.001$ ). In accordance with this, time spent resting alert was lowest 2 h after administration of the challenge ( $31.8 \pm 0.04$  %) ( $P < 0.001$ ). Time spent resting alert was furthermore



**Figure 3.** Feeding motivation of LPS/HuSA challenged birds in response to the challenge. Treatments were combinations of early life feeding strategy (EF = early feeding, DF = delayed feeding) and early life housing conditions (F = floor-housing, C = cage-housing). Data are displayed as means and corresponding SE.

influenced by a combination of feeding strategy and housing conditions ( $P = 0.016$ ). Time spent resting alert in the hours following the challenge was lowest in DF-F birds ( $35.9 \pm 0.04\%$ ) and differed from EF-F birds ( $51.7 \pm 0.04\%$ ) ( $P = 0.048$ ) and DF-C birds ( $52.5 \pm 0.04\%$ ) ( $P = 0.048$ ). Active behavior tended to be lowest 2 h after administration of the challenge ( $P = 0.089$ ).



**Figure 4.** Behavior of LPS/HuSA challenged birds in the hours following the challenge. Treatments were combinations of early life feeding strategy (EF = early feeding, DF = delayed feeding) and early life housing conditions (F = floor-housing, C = cage-housing). Data are displayed as mean percentage of time spent displaying a behavior. An effect of time was found on time spent resting with closed eyes ( $P < 0.0001$ ), with a peak at 2 h after administration of the challenge.

## DISCUSSION

This study investigated the effects of early (EF) and delayed feeding (DF) in different housing conditions (cage vs. floor housing) on BW, the immune response towards an intra-tracheal LPS/HuSA challenge, and the accompanying sickness behavior.

EF birds showed higher feed intake and BW throughout the whole experimental period compared with DF birds, with an average difference in BW of 272 g (2919 g vs. 2647 g) at 42 d of age. Higher BW (Gonzales et al., 2003) and growth (Careghi et al., 2005) in EF birds has been previously observed and may be due to a higher or more optimal nutrient uptake in EF birds, which have an increased intestinal surface area (Uni et al., 1998; Noy et al., 2001; Lamot et al., 2014) and show a higher activity of digestive enzymes (Corless and Sell, 1999).

NAb titers against KLH were not affected by the LPS/HuSA challenge or feeding strategy, but housing conditions had an effect on IgM titers against KLH. Differences between birds of different housing conditions did, however, not exceed 0.6 titer units and further research should elucidate the biological relevance of these differences.

In response to the LPS/HuSA challenge, EF birds showed a higher feed intake as well as a less severe growth check. The most severe growth check was observed in DF-F birds. Interestingly, these birds also showed the highest specific IgY antibody response against HuSA, suggesting that both feeding strategy and antigenic pressure in the environment play a role in immune reactivity. IgM antibody titers were not affected by feeding strategy or housing conditions, indicating that B cell function was not impaired and that treatments or treatment combinations only affected immune reactivity as shown by differences in IgY titers.

A peak in sickness response was observed 2 h after administration of the LPS/HuSA challenge and was most pronounced in DF-F birds. DF-F birds showed the lowest feeding motivation and spent most of the time resting with closed eyes in the hours following the challenge. Two explanations for the most pronounced antibody response and the most pronounced sickness response in DF-F birds are possible. First, Dibner et al. (1998) suggested that DF birds are less capable of handling environmental and disease challenges compared with EF birds. This could

suggest that compared to a cage system, DF is more disadvantageous in a floor system where antigenic pressure is high. The findings of the present study, that DF-F birds showed the most pronounced sickness response after administration of the challenge, might support the notion of Dibner et al. (1998) which is that DF birds have trouble handling environmental challenges. Since the DF-F birds however showed the highest specific antibody response towards HuSA, a second possible explanation is in fact that the opposite is the case, i.e. that these birds are better able to deal with challenges compared to EF birds, but that this comes at the cost of performance.

That feeding strategy as well as environment may influence parts of the immune system has been previously shown, but in contrast to the current study, both factors have so far only been investigated separately from each other. Regarding feeding strategy Bar Shira et al. (2005) for example observed that EF birds showed a more rapid colonization of the hindgut and bursa with B and T lymphocytes and that EF birds showed higher primary antibody responses. Differences in immune reactivity as a consequence of feeding strategy may be caused by differences in intestinal microbial colonization. It has long been known that a dramatic increase in bacterial numbers can be observed upon first feed intake in chickens (Shapiro and Sarles, 1949). Microbiota composition in EF birds might therefore be more complex than that of DF birds, at least during the first 3 d p.h., and it has been demonstrated that a certain complexity in microbial composition is necessary for immune stimulation (Moreau et al., 1978; Rhee et al., 2004). Furthermore it has been shown in humans that initial microbial colonization may have long-lasting effects on microbiota composition (Grönlund et al., 1999). Recently, we found that DF and EF birds indeed differ strongly in their initial ileal microbiota composition, however these differences were diminished after 3 weeks of age (unpublished data). Since adequate microbial colonization is necessary for intestinal development (Tlaskalová-Hogenová et al., 1983; Cebra, 1999), and because microbiota and immune system interact (Peterson et al., 2007; Round and Mazmanian, 2010; Hooper et al., 2012), EF birds may have had a head start over DF birds regarding immune development and the establishment of a balanced immune system. Indeed, deprivation of dietary antigens in early life may lead to a skew towards humoral immune responses (Da Silva Menezes et al., 2003). A skew towards humoral immune responses in DF-F birds, which experienced a delay in

exposure to dietary antigens p.h., is possible, and this would consequently lead to a more pronounced antibody response towards the administered challenge. However, the DF-F birds also show the most pronounced sickness response which is mediated by pro-inflammatory cytokines (Johnson, 1998; Dantzer, 2001), indicating that non-humoral parts of the immune system are affected as well.

There are also indications that colonization with certain types of microbiota in early life may lead to a decreased responsiveness towards LPS (Sjörge et al., 2009), i.e., induction of tolerance, and may influence systemic immunity (Da Silva Menezes et al., 2003; Sjörge et al., 2009). Impaired induction of tolerance by deprivation of antigenic stimulation in early life may also have played a role in DF birds. Klipper et al. (2000) have shown that induction of oral tolerance in chickens is age-dependent and is not possible to achieve after the first week of life, indicating that the first week of life forms a critical window for the imprinting of the immune system. In contrast to EF birds, DF birds in the current study may have passed the most sensitive part of the critical window in which induction of tolerance is still possible by denying them access to antigenic stimulation via diet for the first 3 d p.h. Which mechanisms lie behind the more pronounced antibody response towards HuSA in DF birds remains to be determined, but it is noteworthy that the contrast established in the first 3 d p.h. between EF and DF birds could obviously still affect systemic immunity later in life.

The observed effect of feeding strategy on the systemic humoral immune response only holds for F birds and not for C birds, however, and aside from exposure to dietary antigens, antigenic pressure from the environment seems to play a role in immune reactivity as well. Again, early life conditions seem to be crucial, as shown in a study by Moe et al. (2010), in which rearing conditions, but not housing conditions after the rearing period, influenced antibody responses. Although dust concentrations per m<sup>3</sup> air and endotoxin concentrations in settled dust did not differ much between housing systems probably due to the low density of birds (data not shown), F birds nonetheless face more antigenic pressure from the environment as they are in more direct contact with their feces and therefore conserved microbial molecular patterns such as endotoxins. DF birds who potentially have not built up antigenic tolerance are required to respond to this situation with high immune reactivity towards antigenic challenges. In high density

situations, feeding strategy and early life housing conditions may therefore be of great importance for immune development.

Another possible reason for the observation that feeding strategy seems to play a less important role in C birds may be immune suppression caused by higher stress levels in these birds (Wang et al., 2003), which do not have the possibility to express all of their natural behaviors such as dust bathing and foraging.

In conclusion, early life conditions were shown to have long-lasting effects on systemic humoral immunity in chickens and effects on other parts of the immune system are likely. Immune reactivity later in life may be influenced by feeding strategy immediately p.h. as well as early life housing conditions, where EF seems to be of more importance in a floor-system. These findings stress the importance of management and housing on immune reactivity and the balance between disease resistance and performance, and may have important implications regarding health, including vaccine efficacy, since vaccines may be less or more efficient under certain housing conditions and feeding strategies. Post-hatch feeding strategy as well as early life housing conditions should therefore be taken into account when measuring immune responses and vaccine efficacy in chickens.

### ACKNOWLEDGEMENTS

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## REFERENCES

- Bar Shira, E., D. Sklan, and A. Friedman. 2005. Impaired immune responses in broiler hatchling hindgut following delayed access to feed. *Vet. Immunol. Immunopathol.* 105:33-45.
- Careghi, C., K. Tona, O. Onagbesan, J. Buyse, E. Decuypere, and V. Bruggeman. 2005. The effects of the spread of hatch and interaction with delayed feed access after hatch on broiler performance until seven days of age. *Poult. Sci.* 84:1314-1320.
- Cebra, J. J. 1999. Influences of microbiota on intestinal immune system development. *Am. J. Clin. Nutr.* 69:1046S-1051S.
- Corless, A. B., and J. L. Sell. 1999. The effects of delayed access to feed and water on the physical and functional development on the digestive system of young turkeys. *Poult. Sci.* 78:1158-1169.
- Da Silva Menezes, J., D. De Sousa Mucida, D. Carmona Cara, J. Isaura Alvarez-Leite, M. Russo, N. Monteiro Vaz, and A. M. Caetano de Faria. 2003. Stimulation by food proteins plays a critical role in the maturation of the immune system. *International Immunology* 15:447-455.
- Dantzer, R. 2001. Cytokine-induced sickness behavior: where do we stand? *Brain, Behavior, and Immunity* 15:7-24.
- Dibner, J. J., C. D. Knight, M. L. Kitchell, C. A. Atwell, A. C. Downs, and F. J. Ivey. 1998. Early feeding and development of the immune system in neonatal poultry. *J Appl Poult Res* 7:425-436.
- Ellen, H. H., R. W. Bottcher, E. v. Wachenfelt, and H. Takai. 2000. Dust levels and control methods in poultry houses. *Journal of Agricultural Safety and Health* 6:275-282.
- Geuking, M. B., J. Cahenzli, M. A. E. Lawson, D. C. K. Ng, E. Slack, S. Hapfelmeier, K. D. McCoy, and A. J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 34:794-806.
- Gonzales, E., N. Kondo, É. S. P. B. Saldanha, M. M. Loddy, C. Careghi, and E. Decuypere. 2003. Performance and physiological parameters of broiler chickens subjected to fasting on the neonatal period. *Poult. Sci.* 82:1250-1256.
- Grönlund, M.-M., O.-P. Lehtonen, E. Eerola, and P. Kero. 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *Journal of Pediatric Gastroenterology & Nutrition* 28:19-25.
- Hall, J. A., N. Bouladoux, C. M. Sun, E. A. Wohlfert, R. B. Blank, Q. Zhu, M. E. Grigg, J. A. Berzofsky, and Y. Belkaid. 2008. Commensal DNA limits regulatory T cell

- conversion and is a natural adjuvant of intestinal immune responses. *Immunity* 29:637-649.
- Hapfelmeier, S., M. A. E. Lawson, E. Slack, J. K. Kirundi, M. Stoel, M. Heikenwalder, J. Cahenzli, Y. Velykoredko, M. L. Balmer, K. Endt, M. B. Geuking, R. Curtiss, K. D. McCoy, and A. J. Macpherson. 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* 328:1705-1709.
- He, B., W. Xu, P. A. Santini, A. D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D. M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. *Immunity* 26:812-826.
- Hooper, L. V., D. R. Littman, and A. J. Macpherson. 2012. Interactions between the microbiota and the immune system. *Science* 336:1268-1273.
- Johnson, R. W. 1998. Immune and endocrine regulation of food intake in sick animals. *Domestic Animal Endocrinology* 15:309-319.
- Just, N., S. Kirychuk, Y. Gilbert, V. Létourneau, M. Veillette, B. Singh, and C. Duchaine. 2011. Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations. *Environmental Research* 111:492-498.
- Klipper, E., D. Sklan, and A. Friedman. 2000. Immune responses of chickens to dietary protein antigens I. Induction of systemic and intestinal immune responses following oral administration of soluble proteins in absence of adjuvant. *Vet. Immunol. Immunopathol.* 74:209-223.
- Lamot, D., I. B. Van De Linde, R. Molenaar, C. W. Van der Pol, P. J. A. Wijtten, B. Kemp, and H. Van Den Brand. 2014. Effects of moment of hatch and feed access on chicken development. *Poult. Sci.* 93:1-11.
- Le Bouquin, S., A. Huneau-Salaün, D. Huonnic, L. Balaine, S. Martin, and V. Michel. 2013. Aerial dust concentration in cage-housed, floor-housed, and aviary facilities for laying hens. *Poult. Sci.* 92:2827-2833.
- Mazmanian, S. K., C. H. Liu, A. O. Tzianabos, and D. L. Kasper. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122:107-118.
- Moe, R. O., D. Guémené, M. Bakken, H. J. S. Larsen, S. Shini, S. Lervik, E. Skjerve, V. Michel, and R. Tauson. 2010. Effects of housing conditions during the rearing and laying period on adrenal reactivity, immune response and heterophil to lymphocyte (H/L) ratios in laying hens. *Animal* 4:1709-1715.

- Moreau, M. C., R. Ducluzeau, D. Guy-Grand, and M. C. Muller. 1978. Increase in the Population of Duodenal Immunoglobulin A Plasmocytes in Axenic Mice Associated with Different Living or Dead Bacterial Strains of Intestinal Origin. *Infect. Immun.* 21:532-539.
- Noy, Y., A. Geyra, and D. Sklan. 2001. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poult. Sci.* 80:912-919.
- Peterson, D. A., N. P. McNulty, J. L. Guruge, and J. I. Gordon. 2007. IgA Response to Symbiotic Bacteria as a Mediator of Gut Homeostasis. *Cell Host Microbe* 2:328-339.
- Rhee, K.-J., P. Sethupathi, A. Driks, D. K. Lanning, and K. L. Knight. 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J. Immunol.* 172:1118-1124.
- Round, J. L., and S. K. Mazmanian. 2010. Inducible Foxp3<sup>+</sup> regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc. Natl. Acad. Sci. U.S.A.* 107:12204-12209.
- Shapiro, S. K., and W. B. Sarles. 1949. Microorganisms in the intestinal tract of normal chickens. *J. Bacteriol.* 58:531-544.
- Simon, K., G. De Vries Reilingh, B. Kemp, and A. Lammers. 2014. Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers. *Poultry Science* 93:1-11.
- Sjörgen, Y. M., S. Tomicic, A. Lundberg, M. F. Böttcher, B. Björkstén, E. Sverremark-Ekström, and M. C. Jenmalm. 2009. Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses. *Clinical & Experimental Allergy* 39:1842-1851.
- Tlaskalová-Hogenová, H., J. Šterzl, R. Štěpánková, and V. Dlabáč. 1983. Development of immunological capacity under germfree and conventional conditions. *Annals of the New York Academy of Sciences* 409:96-113.
- Uni, Z., S. Ganot, and D. Sklan. 1998. Posthatch development of mucosal function in the broiler small intestine. *Poult. Sci.* 77:75-82.
- Wang, W., R. F. J. Wideman, M. E. Chapman, T. K. Bersi, and G. F. Erf. 2003. Effect of intravenous endotoxin on blood cell profiles of broilers housed in cages and floor litter environments. *Poult. Sci.* 82:1886-1897.

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# CHAPTER 4

## Long-term effects of early life microbiota disturbance on adaptive immunity in laying hens

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## ABSTRACT

Due to an interplay between intestinal microbiota and immune system, disruption of intestinal microbiota composition during immune development may have consequences for immune responses later in life. The present study investigated the effects of antibiotic treatment in the first weeks of life on the specific antibody response later in life in chickens. Layer chicks received an antibiotic cocktail consisting of vancomycin, neomycin, metronidazole and amphotericin-B by oral gavage every 12 h, and ampicillin and colistin in drinking water for the first week of life. After the first week of life, chicks received ampicillin and colistin in drinking water for two more weeks. Control birds received no antibiotic cocktail and plain drinking water. Fecal microbiota composition was determined during antibiotic treatment (d 8 and 22), two weeks after cessation of antibiotic treatment (d 36) and at the end of the experimental period at d 175 using a 16S ribosomal RNA gene targeted microarray, the ChickChip. During antibiotic treatment fecal microbiota composition differed strongly between treatment groups. Fecal microbiota of antibiotic treated birds consisted mainly of *Proteobacteria*, and in particular *E.coli*, while fecal microbiota of control birds consisted mainly of *Firmicutes*, such as lactobacilli and clostridia. Two weeks after cessation of antibiotic treatment fecal microbiota composition of antibiotic treated birds had recovered and was similar to that of control birds. On d 105, 12 weeks after cessation of antibiotic treatment, chicks of both treatment groups received an intra-tracheal LPS/HuSA challenge. Antibody titers against LPS and HuSA were measured 10 days after administration of the challenge. While T cell independent antibody titers (LPS) were not affected by antibiotic treatment, antibiotic treated birds showed lower T cell dependent antibody titers (HuSA) compared with control birds. In conclusion, intestinal microbial dysbiosis early in life may still have effects on the specific antibody response months after cessation of antibiotic treatment and despite an apparent recovery in microbiota composition.

**Key words:** chicken, antibiotics, microbiota, immune response

## INTRODUCTION

Use of antibiotics severely disrupts the intestinal microbiota composition (Janczyk et al., 2007; Sekirov et al., 2008). Disruption of intestinal microbiota composition may be long-lasting (Jernberg et al., 2007; Schokker et al., 2015) and may already be caused by a single antibiotic dose (Janczyk et al., 2007; Schokker et al., 2015). Even after cessation of antibiotic treatment the intestinal microbiota often does not entirely return to its pre-treatment composition, but is changed persistently (Dethlefsen and Relman, 2011). Due to the constant interaction between microbiota and host immune system, changes in microbiota composition may affect host immunity. A balanced microbiota is important for maintaining the balance between Th1, Th2, Th17 and regulatory T cells (Mazmanian et al., 2005; Ivanov et al., 2008) and helps protect the host from invasion by enteric pathogens. Protection can result from competition for nutrients and binding sites, release of antimicrobial molecules directed against a potential pathogen, and stimulation of host defenses such as secretion of sIgA, defensins, or mucus (Stecher and Hardt, 2011). Furthermore bacterial signaling has been shown to be involved in pro- and anti-inflammatory processes (Willing et al., 2011) and continuous modulation of the innate immune system by microbiota facilitates rapid responses against invading pathogens (Clarke et al., 2010). Disruption of the intestinal microbiota composition and therefore intestinal homeostasis by antibiotics has been shown to affect host defenses and thus makes the host more prone to infections (Clarke et al., 2010; Willing et al., 2011).

The importance of adequate microbial colonization for the establishment and maintenance of a balanced immune system, but also for immune development, has been shown in studies with germ-free animals, in which absence of microbiota entailed compromised immune functions. Germ-free animals showed, for instance, few plasma cells in the small intestine, decreased IgA levels, and under-developed mesenteric lymph nodes and Peyer's patches. These animals furthermore showed low amounts of systemic immunoglobulins and plasma cells, deviant antibody responses, and were more susceptible to various infections (Smith et al., 2007; Sekirov et al., 2008; Clarke et al., 2010; Willing et al., 2011). Many of these impairments in immune function may, however, be restored upon microbial colonization (Smith et al., 2007). Effects of microbial colonization include

induction of regulatory T cell responses (Geuking et al., 2011), increase of intestinal IgA plasma cells (Moreau et al., 1978), and activation and terminal differentiation of B cells (Bos et al., 1987). Intestinal microbiota is furthermore involved in class switching of B cells (He et al., 2007) and development of the mucosal antibody repertoire (Hapfelmeier et al., 2010).

Thus, due to the interaction between microbiota and host immune system antibiotic treatment early in life may have consequences for immune responses later in life. Antibiotic treatment in the present study was based on the protocol of Reikvam et al. (2011), which in mice leads to a phenotype resembling that of germ-free animals. To the best knowledge of the authors this is the first time that the protocol of (Reikvam et al., 2011) has been adapted for use in chickens. The present study aimed at investigating the effects of intestinal microbiota depletion by broad spectrum antibiotics in the first week of life on microbiota composition and on the antibody response towards a non-infectious lung challenge later in life.

## **MATERIALS AND METHODS**

### ***Chickens and Housing***

In this study 40 one-day-old Lohman Brown hens were obtained from a commercial hatchery (Verbeek Hatchery, Zeewolde, The Netherlands) and were randomly divided over two treatment groups: an antibiotic treated group and a control group. D 0 is defined as the day of pull in the present study. Birds were housed in groups of 4, resulting in 5 replicates per treatment group. Birds were housed in floor pens of 2 m<sup>2</sup> containing wood shavings with a regimen of 16L:8D. Both treatment groups had ad libitum access to feed and water. Feed for both treatment groups consisted of a commercial diet containing a coccidiostat (diclazuril).

Antibiotic treatment was adapted from the protocol of Reikvam et al. (2011). Antibiotic treated birds received an antibiotic cocktail (10 ml/kg BW) consisting of vancomycin (5 mg/ml), neomycin (10 mg/ml), metronidazole (10 mg/ml) and amphotericin-B (0.1 mg/ml) for the first 7 d. The antibiotic cocktail was administered by gavage every 12 h. Additionally antibiotic treated birds received

ampicillin (1 g/l) and colistin (200 mg/l) in their drinking water for the first 21 d in order to prevent uncontrolled colonization of pathogens after the severe antibiotic treatment of the first 7 d. Control birds received a placebo (PBS) by gavage every 12 h during the first 7d and plain drinking water. Administration of antibiotics during the first 7 d is defined as first phase antibiotics. Administration of antibiotics from d 8 through d 21 is defined as second phase antibiotics.

At d 105 all birds received a non-infectious lung challenge consisting of intra-tracheally administered *Escherichia coli* lipopolysaccharide (**LPS**) (0.5 mg/kg) (LPS from *E. coli* 055:B5, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, catalog L2880) in combination with Human Serum Albumin (**HuSA**) (0.1 mg/kg) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, catalog A3782).

Chickens were vaccinated against Marek's disease (at hatch), Newcastle disease (at d 8, d 34, d 38), infectious bursal disease (at d 29), infectious laryngotracheitis (at d 91), and infectious bronchitis (at d 113).

This study was approved by the Animal Welfare Committee of Wageningen University and Research Centre in accordance with Dutch laws and regulations on the execution of animal experiments.

### ***Measurements and Sampling***

Birds were weighed daily during the first 7 d post-hatch (**p.h.**) when the antibiotic cocktail was administered and thereafter once a week. Additionally birds were weighed on the day of the immunological challenge (d 105) and on d 1 and 2 after the challenge.

Blood samples were collected from the wing vein at different time points.

On d 35, 70, 105 (prior to administration of the LPS/HuSA challenge), and 115 natural antibody (**NAb**) titers against keyhole limpet hemocyanin (**KLH**) were determined as a measure for the first line of defense. Antibody titers against LPS and HuSA were determined on d 105 (prior to administration of the LPS/HuSA challenge) and d 115. Antibody titers were determined by ELISA as described earlier (Simon et al., 2014) and titers were calculated as described by De Koning et al. (2015).

Total Ig plasma concentrations were determined on d 35, 70, 105, 140, and 175 by means of chicken IgM and IgG ELISA quantitation sets according to

manufacturer's recommendations (Bethyl Laboratories, Inc, Montgomery, TX, USA, catalog E30-102 and E30-104, respectively). Dilution of the HRP detection antibody was adjusted to 1:20,000.

Fecal samples were collected in the morning of d 8, 22, 36, and 175. Pooled samples were collected per pen by placing all 4 birds of each pen in a clean plastic crate for half an hour and collecting the feces afterwards. Pooled fecal samples were mixed thoroughly and stored at -80°C until further use.

### ***Cultivation of Fecal Bacteria***

Part of the pooled fecal samples of d 8 were used directly for cultivation of bacteria in order to determine the amount of cultivable bacteria in feces after the severe antibiotic treatment of the first week. For this purpose 10 mg of pooled fecal samples were dissolved in 1 ml brain-heart infusion. Subsequently, 50  $\mu$ l of sample dilution were added to the first well of a microtiter plate containing 100  $\mu$ l brain-heart infusion. Samples were further diluted across wells with a 3x dilution factor until a dilution of  $2.8 \times 10^{11}$  was reached. After incubation for 24 h at 37°C the last positive well was used for calculation of the number of cultivable bacteria in feces.

### ***Microbiota Analysis***

Microbial DNA was extracted from  $0.25 \pm 0.01$  g feces by using Repeated Bead Beating as described by (Salonen et al., 2010). DNA samples were analyzed by using the Chicken Intestinal Tract Chip (**ChickChip**). The ChickChip is a phylogenetic microarray with more than 2,000 oligonucleotides based on 16S rRNA gene sequences of 567 chicken intestinal microbial species-level phylotypes. Design of the ChickChip was according to the principles described for the Human-Intestinal Tract Chip (**HITChip**) and the hybridization protocol and data analysis were carried out as described for the HIT- and Pig-Intestinal Tract Chip (Rajilić-Stojanović et al., 2009; Haenen et al., 2013). Briefly, bacterial 16S rRNA was amplified using the primers T7prom-Bact-27-for: 5'-TGA ATT GTA ATA CGA CTC ACT ATA GGG GTT TGA TCC TGG CTC AG-3' and Uni-1492-rev: 5'-CGG CTA CCT TGT TAC GAC-3'. PCR products were transcribed into RNA. Purified resultant RNA was labelled with fluorescent CyDye (GE Healthcare Life Sciences, Eindhoven, The Netherlands) and labelled RNA was hybridized to the

array. Microarray images were processed using Agilent's Feature Extraction Software (version 9.1, Agilent Technologies, Amstelveen, The Netherlands). Normalization and further processing of data were performed as described in previous studies (Rajilić-Stojanović et al., 2009; Haenen et al., 2013). Shannon's diversity index was calculated based on probe-level profiles in order to determine differences in microbiota diversity between antibiotic treated and control birds.

### ***Statistical Analysis***

Data were analyzed in SAS 9.2 (SAS Institute Inc., Cary, NC). A mixed linear model was used for data on BW, growth, antibody titers, and microbiota composition (phylum level and approximate genus level). Observations cannot be considered independent, since several measurements on BW, growth, and antibody titers were performed per bird and several fecal samples were taken per pen over time. Therefore a repeated measures analysis was incorporated in the statistical model. Pen was considered the experimental unit. For BW, growth, and antibody titer analysis data was obtained from individual birds and pen was included in the model as random effect. Fecal samples for microbiota analysis were not obtained from individual birds, but were pooled samples per pen and data were therefore already on pen level. Treatment (antibiotic treated, control), time (day p.h.), and their interaction were analyzed for fixed effects on BW, growth, antibody titers, and microbiota composition. The effect of treatment (antibiotic treated, control) on the number of cultivable bacteria in feces on d 8 and on microbiota diversity was tested with a mixed linear model.

A natural log transformation was performed on data on microbiota composition and number of cultivable bacteria in feces to approximate a normal distribution. Normality was checked on the residuals. Data are displayed as means and corresponding SE. Differences are considered significant where  $P \leq 0.05$ .

## RESULTS

### *BW*

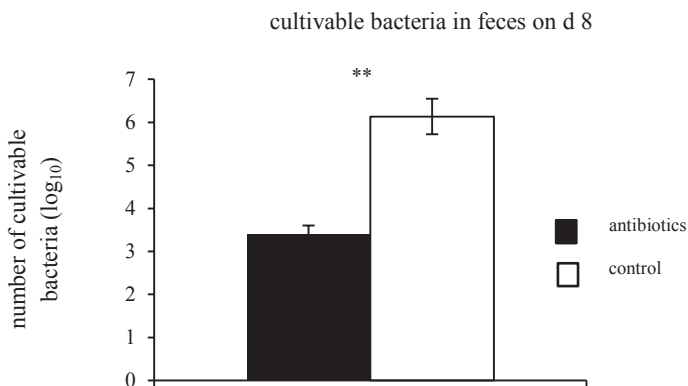
BW was neither during administration of the first phase antibiotics in the first 7 d p.h. nor during the rest of the experimental period affected by administration of antibiotics (data not shown).

### *Cultivable Bacteria in Feces*

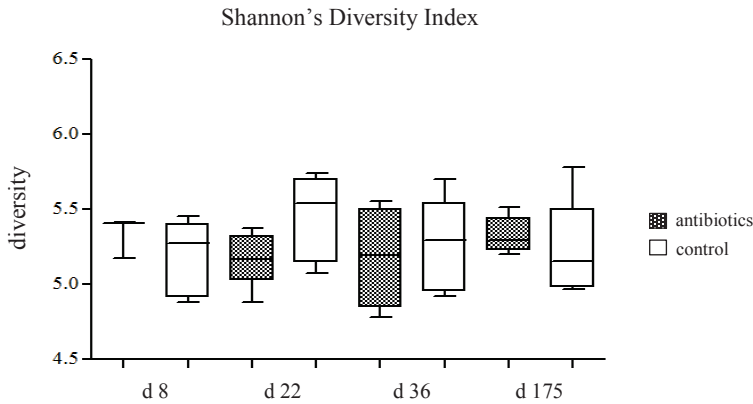
The total number of cultivable bacteria in feces on d 8 p.h. (Figure 1), i.e. the end of administration of the antibiotic cocktail was on average  $2.7 \times 10^3$  times lower in antibiotic treated birds compared with control birds ( $P < 0.01$ ).

### *Microbiota Composition*

Microbiota diversity did not differ between antibiotic treated and control birds (figure 2).



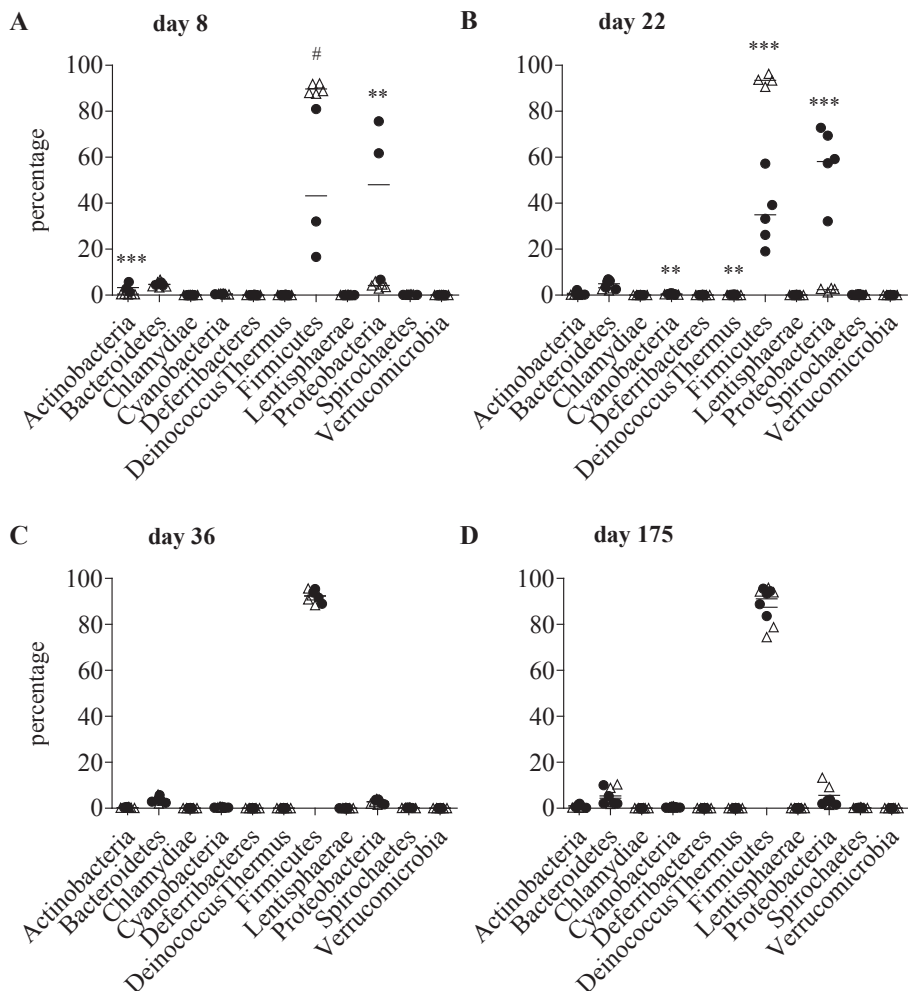
**Figure 1.** Number of cultivable bacteria in feces of antibiotic treated and control birds on day 8 post hatch. Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 through day 7 post hatch. Due to extremely low numbers of cultivable bacteria in feces of antibiotic treated birds the left bar is barely visible in the graph. Data are displayed as means and corresponding SE. \*\* = significant difference ( $P \leq 0.01$ ) between antibiotic treated and control birds.



**Figure 2.** Shannon's diversity index for antibiotic treated and control birds on different days post hatch based on ChickChip oligonucleotide probe signals. Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 trough day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. Data are displayed as means and corresponding SE.

At phylum level (Figure 3) *Actinobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, and *Proteobacteria* were influenced by a combination of treatment and time ( $P < 0.01$  for all). It should be noted that on d 8 microbiota in two antibiotic treated pens had been depleted to an extent that no bacterial DNA could be retrieved for analysis of microbiota composition. Differences in fecal microbiota composition between antibiotic treated and control birds were most pronounced regarding *Proteobacteria* and *Firmicutes*. At the end of first phase antibiotics on d 8, antibiotic treated birds showed a higher abundance of *Proteobacteria* ( $48.0 \pm 21.0$  %) and a lower abundance of *Firmicutes* ( $43.2 \pm 19.4$  %) compared with control birds ( $4.2 \pm 0.4$  % and  $89.7 \pm 1.0$  %, respectively) ( $P = 0.004$  and  $P = 0.06$ , respectively). At the end of second phase antibiotics, antibiotic treated birds still showed a higher abundance of *Proteobacteria* ( $58.2 \pm 7.1$  %) and a lower abundance of *Firmicutes* ( $35.0 \pm 6.5$  %) compared with control birds ( $2.4 \pm 0.4$  % and  $93.5 \pm 1.2$  %, respectively) ( $P < 0.001$  for both).

Less pronounced differences between treatment groups included a higher relative abundance of *Actinobacteria* in antibiotic treated birds ( $3.3 \pm 1.3$  %) compared with control birds ( $0.4 \pm 0.05$  %) ( $P < 0.001$ ) on d 8. Furthermore,

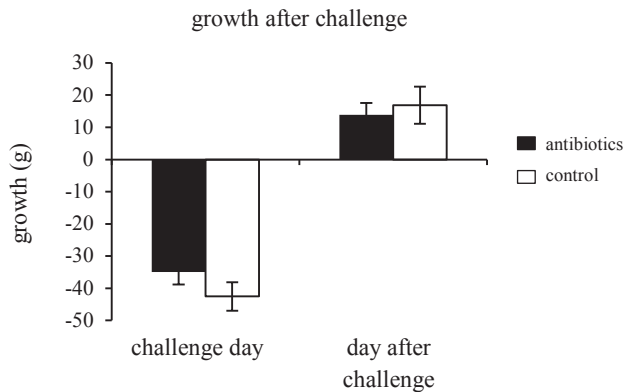


**Figure 3.** Fecal microbiota composition assessed at phylum level for antibiotic treated (full circles) and control birds (open triangles). Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 trough day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. Horizontal bars represent the mean. Significant differences between treatment groups are indicated as follows: \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ , # =  $P \leq 0.1$ .

antibiotic treated birds showed higher relative abundances of *Cyanobacteria* ( $0.6 \pm 0.1\%$ ) compared with control birds ( $0.3 \pm 0.1\%$ ) ( $P = 0.01$ ) on d 22. On that day relative abundance of *Deinococcus-Thermus* was also higher in antibiotic treated birds ( $0.16 \pm 0.02\%$ ) compared with control birds ( $0.04 \pm 0.004\%$ ) ( $P < 0.001$ ).

No effects of antibiotic treatment on the relative abundance of *Bacteroidetes*, *Chlamydiae*, *Deferribacteres*, *Lentisphaerae*, *Spirochaetes*, and *Verrucomicrobia* were found. No differences at phylum level between antibiotic treated birds and control birds were observed on d 36 and d 175.

The differences in the relative abundance of bacteria between treatment groups that underlie the differences found at phylum level become clear when zooming in on approximate genus level where several statistically significant differences between antibiotic treated and control birds were observed on d 8 and d 22 (Table 1). Most striking was the fact that on d 22 fecal samples of antibiotic treated birds consisted on average of 53 % ( $\pm 7.1$  %) *E. coli* et rel., while this group accounted for less than 1 % of fecal bacteria in control birds. Other potential pathogens, such as *Pasteurella* et rel., *Pseudomonas* et rel., or *Bordetella* et rel. were also present in a higher relative abundance in antibiotic treated birds, while potentially beneficial bacteria such as different lactobacilli and several members of the class *Clostridia* were generally more abundant in control birds. No significant differences at approximate genus level between treatment groups were found for d 36 or d 175.



**Figure 4.** Growth of antibiotic treated and control birds in response to an intra-tracheally administered LPS/HuSA challenge. Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 through day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. The challenge was administered at day 105 post hatch. Data are displayed as means and corresponding SE.

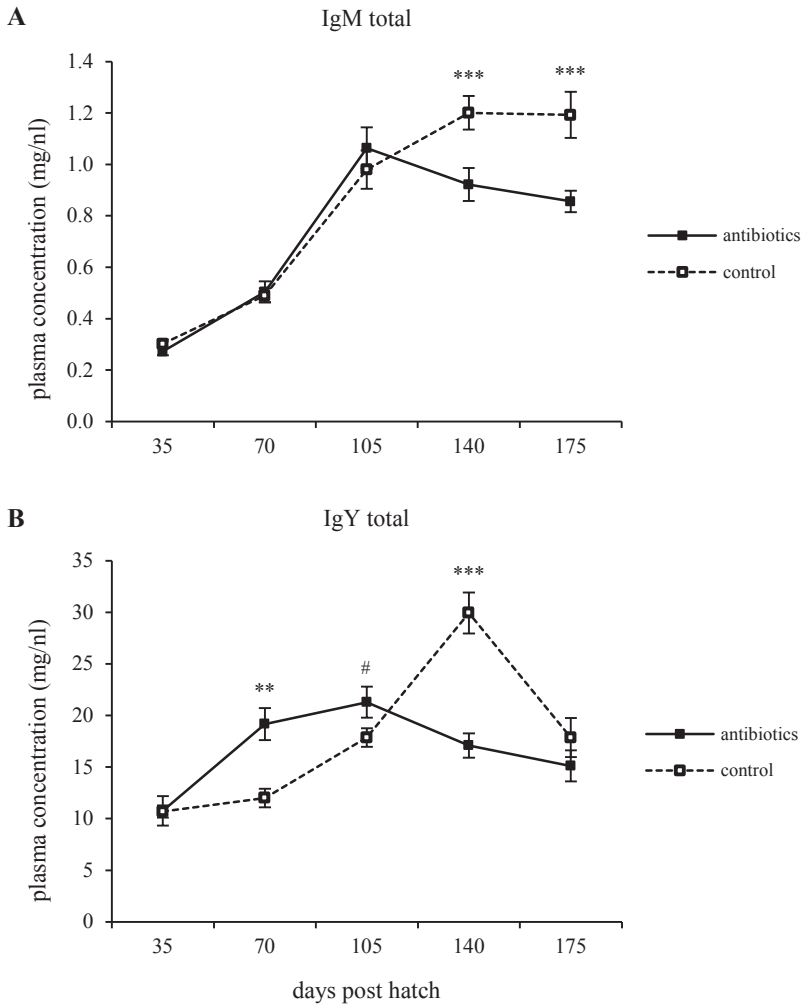
### ***Weight Loss after LPS/HuSA Challenge***

On the day of administration of the LPS/HuSA challenge, birds of both treatment groups showed a loss of BW, on average between 35 g (antibiotic treated) to 43 g (control). On the day after challenge birds of both treatment groups started to gain weight again. Antibiotic treatment did not affect BW loss in response to the administered challenge (Figure 4).

### ***Total IgM & IgY Plasma Concentration***

Total IgM plasma concentration (Figure 5A) was influenced by a combination of antibiotic treatment and time ( $P < 0.001$ ). On d 140 antibiotic treated birds had a lower concentration of total IgM in plasma ( $0.9 \pm 0.06$  mg/ml) compared to control birds ( $1.2 \pm 0.06$  mg/ml) ( $P = 0.001$ ). Total IgM plasma concentration was still lower in antibiotic treated birds on d 175 ( $0.9 \pm 0.04$  mg/ml) compared with control birds ( $1.2 \pm 0.09$  mg/ml) ( $P < 0.001$ ).

Total IgY plasma concentration (Figure 5B) was influenced by a combination of antibiotic treatment and time ( $P < 0.001$ ). On d 63 antibiotic treated birds had a higher concentration of total IgY in plasma ( $19.2 \pm 1.6$  mg/ml) compared with control birds ( $12.0 \pm 0.9$  mg/ml) ( $P = 0.001$ ). Total IgY plasma concentration still tended to be higher in antibiotic treated birds on d 105 ( $21.3 \pm 1.5$  mg/ml) compared with control birds ( $17.9 \pm 0.9$  mg/ml) ( $P = 0.1$ ). On d 140 total IgY plasma concentration was lower in antibiotic treated birds ( $17.1 \pm 1.2$  mg/ml) compared with control birds ( $29.9 \pm 2.0$  mg/ml) ( $P < 0.001$ ).



**Figure 5.** Total IgM (A) and IgY (B) plasma concentrations of antibiotic treated and control birds on different days post hatch. Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 through day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. Data are displayed as means and corresponding SE. \*\* = significant difference ( $P \leq 0.01$ ) and \*\*\* = significant difference ( $P \leq 0.001$ ) between antibiotic treated and control birds. # = tendency for a difference ( $P \leq 0.1$ ) between antibiotic treated and control birds.

**Table 1.** Statistically significant differences on approximate genus level between treatment groups

Phylum	Class	Approximate Genus	Day 8	
			Antibiotic	Control
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Corynebacterium</i> et rel. <i>Micrococcus</i> et rel.	2.98 ± 1.23 **	0.30 ± 0.04
<i>Cyanobacteria</i>		Uncultured <i>Cyanobacteria</i>		
<i>Deinococcus-Thermus</i>	<i>Deinococci</i>	<i>Meiothermus</i> et rel.		
<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillus</i> et rel.	3.41 ± 1.75 **	0.69 ± 0.07
		<i>Carnobacterium</i> et rel.	0.70 ± 0.36 **	0.07 ± 0.01
		<i>Enterococcus</i> et rel.		
		<i>Lactobacillus acidophilus</i> et rel.	1.16 ± 0.72 **	13.62 ± 5.47
		<i>Lactobacillus gasseri</i> et rel.	0.82 ± 0.54 **	15.12 ± 5.24
		<i>Lactobacillus kitasatonis</i> et rel.	2.23 ± 2.02 **	10.15 ± 2.92
		<i>Lactobacillus plantarum</i> et rel.		
		<i>Weissella</i> et rel.		
	<i>Clostridia</i>	<i>Anaerotruncus</i> et rel.		
		<i>Clostridium hylemonae</i> et rel.		
		<i>Clostridium leptum</i> et rel.		
		<i>Clostridium perfringens</i> et rel.		
		<i>Clostridium symbiosum</i> et rel.		
		<i>Eubacterium desmolans</i> et rel.		
		<i>Eubacterium hallii</i> et rel.		
		<i>Eubacterium plexicaudatum</i> et rel.		
		<i>Faecalibacterium</i> et rel.		
		<i>Lachnospira pectinoschiza</i> et rel.	0.07 ± 0.03 *	0.17 ± 0.04
		Uncultured <i>Clostridia</i> close to <i>Clostridium symbiosum</i>		
		Uncultured <i>Clostridia XIVa</i>		
		Uncultured <i>Clostridia XIVb</i>	0.73 ± 0.18 †	0.93 ± 0.06
	<i>Coccus</i>	<i>Staphylococcus aureus</i> et rel.	3.55 ± 2.58 **	0.30 ± 0.03
	<i>Mollicutes</i>	<i>Acholeplasma</i> et rel.	0.03 ± 0.007 †	0.05 ± 0.009
<i>Proteobacteria</i>	<i>Alpha-Proteobacteria</i>	<i>Caulobacter</i> et rel.	0.62 ± 0.23 ***	0.07 ± 0.01
	<i>Beta-Proteobacteria</i>	<i>Bordetella</i> et rel.	15.79 ± 7.29 **	0.60 ± 0.05
		<i>Oxalobacter</i> et rel.	1.66 ± 0.69 ***	0.12 ± 0.02
	<i>Gamma-Proteobacteria</i>	<i>Acinetobacter</i> et rel.	7.92 ± 3.83 **	0.22 ± 0.02
		<i>Escherichia coli</i> et rel.	6.29 ± 2.54 †	1.56 ± 0.46
		<i>Pasteurella</i> et rel.		
		<i>Pseudomonas</i> et rel.	1.34 ± 0.45 **	0.25 ± 0.04
		<i>Psychrobacter</i> et rel.	0.33 ± 0.10 **	0.08 ± 0.01
		Uncultured <i>Gamma-Proteobacteria</i>		
		<i>Xanthomonas</i>	12.47 ± 6.02 ***	0.15 ± 0.02

Data displayed are a selection of fecal bacterial groups on approximate genus level, i.e. bacterial groups that differ in their relative abundance between antibiotic treated birds and control birds. Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 trough day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. (continued on the next page)

**Table 1. (continued)** Statistically significant differences on approximate genus level between treatment groups

Phylum	Class	Approximate Genus	Day 22	
			Antibiotic	Control
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Corynebacterium</i> et rel. <i>Micrococcus</i> et rel.		
<i>Cyanobacteria</i>		Uncultured <i>Cyanobacteria</i>	0.58 ± 0.08 *	0.31 ± 0.07
<i>Deinococcus-Thermus</i>	<i>Deinococci</i>	<i>Meiothermus</i> et rel.	0.16 ± 0.01 **	0.04 ± 0.004
<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillus</i> et rel.	0.72 ± 0.09 *	0.50 ± 0.07
		<i>Carnobacterium</i> et rel.	0.08 ± 0.01 *	0.04 ± 0.01
		<i>Enterococcus</i> et rel.		
		<i>Lactobacillus acidophilus</i> et rel.	0.95 ± 0.45 **	16.14 ± 8.63
		<i>Lactobacillus gasseri</i> et rel.	0.74 ± 0.27 **	12.77 ± 5.51
		<i>Lactobacillus kitasatonis</i> et rel.	0.13 ± 0.03 ***	4.64 ± 2.18
		<i>Lactobacillus plantarum</i> et rel.		
		<i>Weissella</i> et rel.		
	<i>Clostridia</i>	<i>Anaerotruncus</i> et rel.		
		<i>Clostridium hylemonae</i> et rel.		
		<i>Clostridium leptum</i> et rel.		
		<i>Clostridium perfringens</i> et rel.		
		<i>Clostridium symbiosum</i> et rel.		
		<i>Eubacterium desmolans</i> et rel.		
		<i>Eubacterium hallii</i> et rel.		
		<i>Eubacterium plexicaudatum</i> et rel.		
		<i>Faecalibacterium</i> et rel.		
		<i>Lachnospira pectinoschiza</i> et rel.		
		Uncultured <i>Clostridia</i> close to <i>Clostridium symbiosum</i>		
		Uncultured <i>Clostridia XIVa</i>		
		Uncultured <i>Clostridia XIVb</i>	4.63 ± 2.32 *	0.73 ± 0.10
	<i>Coccus</i>	<i>Staphylococcus aureus</i> et rel.	0.39 ± 0.08 †	0.21 ± 0.04
	<i>Mollicutes</i>	<i>Acholeplasma</i> et rel.	0.30 ± 0.13 **	0.04 ± 0.005
<i>Proteobacteria</i>	<i>Alpha-Proteobacteria</i>	<i>Caulobacter</i> et rel.		
	<i>Beta-Proteobacteria</i>	<i>Bordetella</i> et rel.	1.01 ± 0.25 *	0.41 ± 0.08
		<i>Oxalobacter</i> et rel.	0.17 ± 0.05 †	0.08 ± 0.02
	<i>Gamma-Proteobacteria</i>	<i>Acinetobacter</i> et rel.	0.32 ± 0.05 **	0.15 ± 0.02
		<i>Escherichia coli</i> et rel.	53.28 ± 7.14 ***	0.55 ± .012
		<i>Pasteurella</i> et rel.	0.83 ± 0.07 ***	0.18 ± 0.04
		<i>Pseudomonas</i> et rel.		
		<i>Psychrobacter</i> et rel.	0.31 ± 0.04 ***	0.05 ± 0.01
		Uncultured <i>Gamma-Proteobacteria</i>	0.10 ± 0.01 ***	0.02 ± 0.004
		<i>Xanthomonas</i>	0.75 ± 0.13 ***	0.12 ± 0.03

When time and treatment showed a significant ( $P \leq 0.05$ ) interaction, significant differences between treatment groups were found for day 8 and day 22 post hatch. Main effects of treatment on bacterial groups of the two treatment groups are displayed under the “whole period” column. Data are displayed as means and corresponding SE. (continued on the next page)

**Table 1. (continued)** Statistically significant differences on approximate genus level between treatment groups

Phylum	Class	Approximate Genus	Whole Period	
			Antibiotic	Control
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Corynebacterium</i> et rel. <i>Micrococcus</i> et rel.	0.09 ± 0.02 *	0.05 ± 0.01
<i>Cyanobacteria</i>		Uncultured <i>Cyanobacteria</i>		
<i>Deinococcus-Thermus</i>	<i>Deinococci</i>	<i>Meiothermus</i> et rel.		
<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillus</i> et rel. <i>Carnobacterium</i> et rel. <i>Enterococcus</i> et rel. <i>Lactobacillus acidophilus</i> et rel. <i>Lactobacillus gasseri</i> et rel. <i>Lactobacillus kitasatonis</i> et rel. <i>Lactobacillus plantarum</i> et rel. <i>Weissella</i> et rel.	5.33 ± 1.47 †      8.34 ± 1.73 * 0.10 ± 0.07 †	2.68 ± 1.01      13.58 ± 2.21 0.03 ± 0.004
	<i>Clostridia</i>	<i>Anaerotruncus</i> et rel. <i>Clostridium hylemonae</i> et rel. <i>Clostridium leptum</i> et rel. <i>Clostridium perfringens</i> et rel. <i>Clostridium symbiosum</i> et rel. <i>Eubacterium desmolans</i> et rel. <i>Eubacterium hallii</i> et rel. <i>Eubacterium plexicaudatum</i> et rel. <i>Faecalibacterium</i> et rel. <i>Lachnospira pectinoschiza</i> et rel. Uncultured <i>Clostridia</i> close to <i>Clostridium symbiosum</i> Uncultured <i>Clostridia XIVa</i> Uncultured <i>Clostridia XIVb</i>	0.36 ± 0.04 † 0.09 ± 0.01 * 0.32 ± 0.04 † 0.17 ± 0.04 * 0.31 ± 0.14 * 0.04 ± 0.003 * 0.10 ± 0.01 † 0.09 ± 0.01 † 0.71 ± 0.13 **  0.34 ± 0.06 * 0.06 ± 0.02 *	0.51 ± 0.09 0.12 ± 0.02 0.65 ± 0.16 0.38 ± 0.10 0.50 ± 0.14 0.06 ± 0.01 0.12 ± 0.01 0.14 ± 0.02 1.02 ± 0.14  0.82 ± 0.23 0.08 ± 0.02
	<i>Coccus</i>	<i>Staphylococcus aureus</i> et rel.		
	<i>Mollicutes</i>	<i>Acholeplasma</i> et rel.		
<i>Proteobacteria</i>	<i>Alpha-Proteobacteria</i>	<i>Caulobacter</i> et rel.		
	<i>Beta-Proteobacteria</i>	<i>Bordetella</i> et rel. <i>Oxalobacter</i> et rel.		
	<i>Gamma-Proteobacteria</i>	<i>Acinetobacter</i> et rel. <i>Escherichia coli</i> et rel. <i>Pasteurella</i> et rel. <i>Pseudomonas</i> et rel. <i>Psychrobacter</i> et rel. Uncultured <i>Gamma-Proteobacteria</i> <i>Xanthomonas</i>		

Symbols indicate a significant difference (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ ) or tendency for a difference († =  $P \leq 0.1$ ) between treatment groups within the respective time point.

### Antibody Titers

Neither IgM nor IgY NAb titers against KLH were influenced by antibiotic treatment, but titers increased over time ( $P < 0.001$ ) (Table 2A).

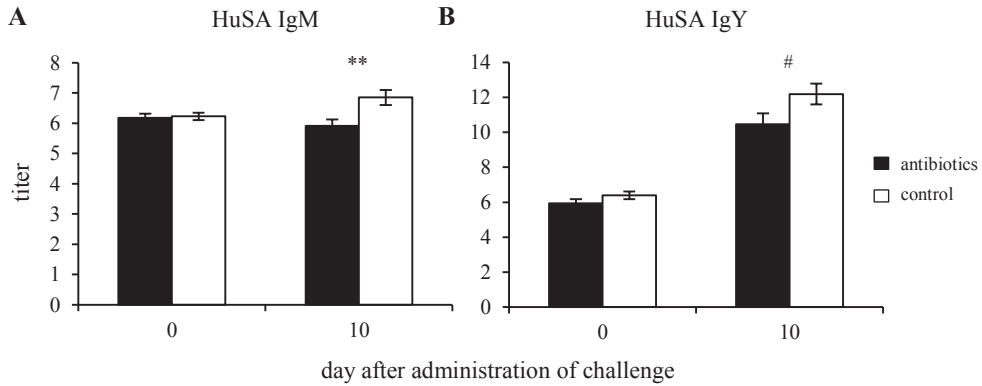
Neither IgM nor IgY antibody titers against LPS were influenced by antibiotic treatment or time (Table 2B).

IgM antibody titers against HuSA (Figure 6A) were influenced by a combination of treatment and time ( $P = 0.01$ ). Ten d after administration of the LPS/HuSA challenge, antibiotic treated birds showed lower IgM titers against HuSA ( $5.9 \pm 0.2$ ) compared with control birds ( $6.9 \pm 0.2$ ) ( $P = 0.007$ ). IgY antibody titers against HuSA (Figure 6B) had increased in both treatment groups 10 d after the LPS/HuSA challenge compared to the day before administration of the challenge ( $P < 0.001$ ). Furthermore antibiotic treated birds tended to have lower IgY antibody titers against HuSA ( $10.5 \pm 0.6$ ) 10 d after the challenge compared with control birds ( $12.2 \pm 0.6$ ) ( $P = 0.08$ ).

**Table 2.** Antibody titers against KLH and LPS.

			Treatment Groups	
day post hatch			antibiotic treated	control
A.	KLH IgM	35	4.9±0.2	4.7±0.2
		70	7.4±0.3	7.2±0.2
		105	9.5±0.3	9.2±0.2
		115	9.0±0.2	9.1±0.2
	KLH IgY	35	4.6±0.3	5.5±0.4
		70	7.6±0.3	6.8±0.3
		105	9.3±0.2	10.0±0.4
		115	9.9±0.3	10.6±0.4
B.	LPS IgM	105	8.7±0.2	8.8±0.2
		115	8.5±0.2	8.5±0.2
	LPS IgY	105	9.8±0.3	9.8±0.2
		115	10.1±0.2	10.0±0.3

Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 through day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. Data are displayed as means and corresponding SE.



**Figure 6.** Specific IgM (A) and IgY (B) antibody titers against HuSA of antibiotic treated and control birds in response to an intra-tracheally administered LPS/HuSA challenge at day 105 post hatch. Antibiotic treated birds received a broad spectrum antibiotic cocktail from day 1 through day 7 post hatch and a milder antibiotic treatment from day 8 through day 21 post hatch. Data are displayed as means and corresponding SE. \*\* = significant difference ( $P \leq 0.01$ ) between antibiotic treated and control birds. # = tendency for a difference ( $P \leq 0.1$ ) between antibiotic treated and control birds.

## DISCUSSION

The present study investigated the effects of broad spectrum antibiotic treatment early in life on BW, cultivable bacteria in feces, fecal microbiota composition, total plasma Ig concentration, NAb titers, and specific antibody titers in response to an intra-tracheal LPS/HuSA challenge later in life. Administration of broad spectrum antibiotics was based on a study by Reikvam et al. (2011), in which mice were depleted of cultivable bacteria in feces by severe antibiotic treatment. In their study administration of a broad spectrum antibiotic cocktail led to a reduction in spleen size, fewer Peyer's patches, enlarged ceca, and a decrease in the expression of antimicrobial factors, leading to a phenotypical resemblance of antibiotic treated mice with germ-free mice.

As expected, at the end of administration of the first phase antibiotics on d 8, antibiotic treated birds showed a considerable reduction in the number of cultivable bacteria in feces, which was on average  $2.7 \times 10^3$  times lower compared with

control birds, confirming that antibiotic treatment was efficient to a similar extent as in the study of Reikvam et al. (2011).

In the present study antibiotic treated birds did not differ in fecal microbiota diversity from control birds. Administration of broad spectrum antibiotics did, however, have a profound effect on fecal microbiota composition. By d 22 antibiotic treated birds showed a shift towards *Proteobacteria*, which accounted for almost 60 % of their fecal microbiota and the vast majority of this group consisted of *E. coli* et rel. Other potential pathogens were also more abundant in antibiotic treated birds, while *Firmicutes* associated with a normal gut microbiota such as certain types of lactobacilli were decreased in antibiotic treated birds. These findings are not surprising, since studies have shown that antibiotic treatment is often accompanied with a loss of colonization resistance, i.e. an increase in opportunistic potential pathogens belonging to *Proteobacteria*, while numbers of potentially beneficial bacteria belonging to *Firmicutes* often decrease (Van Der Waaij et al., 1971; Hengstes et al., 1985; Sullivan and Nord, 2001; Edlund and Nord, 2003; Fouhy et al., 2012). Additionally, administration of antibiotics can lead to increased antibiotic resistance in some bacteria and a subsequent increase in numbers of these bacteria that survive antibiotic treatment (Jakobsson et al., 2010).

Studies in mice and humans show that certain lactobacilli, which were also found to be more abundant in control birds of the present study, may enhance innate and adaptive immunity (Gill et al., 2000; Kawase et al., 2012), direct T cell mediated immune responses (Mohamadzadeh et al., 2005), and attenuate inflammatory processes (Schultz et al., 2002; Osman et al., 2004; Pathmakanthan et al., 2004; Petrof et al., 2009). Furthermore lactobacilli may be able to inhibit growth of potential pathogens such as *E. coli* or *Staphylococcus aureus*, as has been shown for *Lactobacillus plantarum* (Gilliland and Speck, 1977; Gilliland, 1979). Clostridia, some of which were more abundant in control birds of the present study, on the other hand have been found to be important for the maintenance of intestinal homeostasis by playing an important role in the induction of regulatory T cells (Atarashi et al., 2011; Lopetuso et al., 2013). Atarashi et al. (2011) have furthermore shown that treatment with several of the antibiotics used in the present study, leads to a reduction in colonic regulatory T cells in mice in response to administration of antibiotics. Disturbances in microbiota composition by antibiotic treatment may therefore have influenced immune development and

consequently the specific antibody response towards the LPS/HuSA challenge in antibiotic treated birds. The influence of intestinal microbiota composition on immune development and host immunity has extensively been reviewed (e.g. (Round and Mazmanian, 2009; Hill and Artis, 2010; Hooper et al., 2012). Numerous studies have revealed an influence of gut microbiota on the development of various parts of the host immune system, such as the development and function of dendritic cells, macrophages, B cells, T cells, and the maintenance of immune homeostasis.

In contrast to other studies performed in adult humans and developing pigs where disturbances of microbiota composition caused by antibiotic treatment could last up to several months or years (Jernberg et al., 2007; Jakobsson et al., 2010; Dethlefsen and Relman, 2011; Schokker et al., 2015), the fecal microbiota of antibiotic treated birds in the present study resembled that of control birds only two weeks after cessation of antibiotic treatment. It is possible that differences in microbiota composition did not persist in chickens for a longer period of time due to developmental differences between different species. Microbial colonization of antibiotic treated birds was, however, hampered in a period which is seen as a critical window for programming of the immune system (Renz et al., 2012). Different studies have shown that changes in intestinal microbiota composition early in life may have effects on adaptive as well as innate components of the host immune system. For example, changes in microbiota composition early in life have been shown to lead to the development of allergic diseases later in life (Russell et al., 2012) and to affect numbers of invariant natural killer T cells in the intestine (Olszak et al., 2012). Invariant natural killer T cells show characteristics of NK cells as well as T cells and form a bridge between innate and adaptive arms of the immune system (Van Kaer et al., 2011). These effects on the immune system were only found when intestinal microbiota composition was affected early in life, but not when disturbances were induced in adult animals, again emphasizing the importance of early life microbiota composition. In the present study early life antibiotic treatment had long-lasting effects on the specific T cell dependent antibody response (HuSA). Early life antibiotic treatment led to lower antibody titers against HuSA in response to a combination of HuSA and LPS, which was intra-tracheally administered 12 weeks after cessation of antibiotic treatment. Suppression of T cell dependent antibody responses by antibiotic treatment has

also been reported by others (Hauser and Remington, 1982; Woo et al., 1999). Neither antibiotic treated nor control birds reacted to LPS in terms of specific antibodies. Birds of both treatment groups did, however, show a transient growth check in response to the challenge, which did not differ between treatment groups. The transient growth check indicates an innate response towards LPS, since administration of HuSA alone does not lead to a loss in BW (Parmentier et al., 2008). Other studies in chickens have shown differential effects of antibiotics on the antibody response. Murai et al. (2015) found that oral administration of antibiotics leads to enhanced antibody responses against orally administered KLH, but not muscularly administered KLH. The authors furthermore suggested a link between altered antibody responses and a decreased abundance of lactobacilli in feces, since antibody responses were only affected when the antibiotic dose was high enough to affect the abundance of lactobacilli in fecal samples. Khalifeh et al. (2009) on the other hand found that an orally administered antibiotic cocktail lowered antibody responses after a vaccination against Newcastle disease. Brisbin et al. (2008) again found that in-feed antibiotics enhanced antibody responses towards systemically, but not orally administered KLH and no differences in antibody response were found on other orally or systemically administered antigens. Discrepancies between studies indicate that the type and dose of antibiotic as well as duration of antibiotic treatment, route of immunization, and type of antigen may play a role in the way in which antibiotics affect the humoral response in chickens.

A possible explanation for lower antibody responses against HuSA in antibiotic treated birds of the present study might be the massive population with *E.coli* et rel. early in life and consequently the birds' exposure to large amounts of LPS. Although bacterial endotoxins like LPS generally work as adjuvants when administered simultaneously with or shortly after a T cell dependent antigen (Luecke and Sibal, 1962; Merritt and Johnson, 1963; Hamaoka and Katz, 1973), LPS may have a suppressive effect on antibody responses when administered some time before the model antigen (Luecke and Sibal, 1962; Merritt and Johnson, 1963; Persson, 1977). It has been suggested that LPS may directly affect regulatory T cells via the TLR-4 receptor, leading to an enhanced suppressor function of regulatory T cells (Caramalho et al., 2003), which has also been demonstrated in chickens several days after an injection with LPS (Shanmugasundaram and

Selvaraj, 2012). The actions of LPS may also be dose dependent, since a high dose of LPS has been shown to elicit a Th1 response, while a low dose of LPS elicited a Th2 response (Eisenbarth et al., 2002). The exposure to high amounts of LPS during a critical phase in immune development may have shifted immune responses of antibiotic treated birds towards a Th1 type response. Whether this is true and whether the possibly enhanced suppressor function of regulatory T cells is long-lasting remains to be investigated. The considerations regarding a possible shift towards a Th1 type response and enhanced suppressor functions of regulatory T cells may also explain the observed lower IgM and IgY plasma levels in antibiotic treated birds in the period after administration of the LPS/HuSA challenge at d 105 and two vaccinations in the same period.

Apart from total IgM and IgY plasma concentrations, NAb titers against KLH were measured as an indicator for the general immune status of birds. NAb titers, which are part of the first line of defense were not affected by antibiotic treatment at the selected time points. The first blood collection took place on d 35, 2 weeks after cessation of antibiotic treatment. It is therefore not known whether NAb levels against KLH were influenced during antibiotic treatment. NABs are considered an evolutionary preserved element of the immune system and are vital in the first line of defense (Avrameas, 1991; Ochsenbein et al., 1999). Due to their vital role in the defense against pathogens it may be possible that NAb levels were either unaffected by antibiotic treatment or quickly recovered after cessation of antibiotic treatment.

Taken together, the present study shows that antibiotic treatment in the first weeks of life can still affect the specific antibody response and total IgM and IgY plasma concentrations months after cessation of antibiotic treatment, despite an apparent recovery of fecal microbiota composition. Results of the present study reinforced that a dysbiosis of intestinal microbiota early in life potentially leads to alterations in immune development and consequently immune competence in the long run. It should be kept in mind that the present study was conducted with healthy chickens and a relatively mild immunological challenge. It would be interesting to investigate the consequences of early life microbial dysbiosis under more challenging conditions closer to practice, i.e. higher levels of antigenic pressure and stress.

## REFERENCES

- Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, Y. Taniguchi, K. Takeda, S. Hori, I. I. Ivanov, Y. Umesaki, K. Itoh, and K. Honda. 2011. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* 331:337-341.
- Avrameas, S. 1991. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol. Today* 12:154-159.
- Bos, N. A., C. G. Meeuwse, H. Hooijkaas, R. Benner, B. S. Wostmann, and J. R. Pleasants. 1987. Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. *Cell. Immunol.* 105:235-245.
- Brisbin, J. T., J. Gong, C. A. Lusty, P. Sabour, B. Sanei, Y. Han, P. E. Shewen, and S. Sharif. 2008. Influence of in-feed virginimycin on the systemic and mucosal antibody response of chickens. *Poult. Sci.* 87:1995-1999.
- Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express Toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 197:403-411.
- Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16:228-231.
- De Koning, D. B., E. P. C. W. Damen, M. G. B. Nieuwland, E. M. Van Grevenhof, W. Hazeleger, B. Kemp, and H. K. Parmentier. 2015. Association of natural (auto-) antibodies in young gilts with osteochondrosis at slaughter. *Livest. Sci* 176:152-160.
- Dethlefsen, L., and D. A. Relman. 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci. U.S.A.* 108:4554-4561.
- Edlund, C., and C.-E. Nord. 2003. Ecological impact of antimicrobial agents on human intestinal microflora. *Old Herborn University Seminar Monograph 7: Immune system and microflora*; eds. Heidt, P.J., Rusch, V., Van der Waaij, D.; Herborn Litterae:37-65.
- Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 196:1645-1651.
- Fouhy, F., C. M. Guinae, S. Hussey, R. Wall, C. A. Ryan, E. M. Dempsey, B. Murphy, R. P. Ross, G. F. Fitzgerald, C. Stanton, and P. D. Cotter. 2012. High-throughput

- sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob. Agents Chemother.* 56:5811-5820.
- Geuking, M. B., J. Cahenzli, M. A. E. Lawson, D. C. K. Ng, E. Slack, S. Hapfelmeier, K. D. McCoy, and A. J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 34:794-806.
- Gill, H. S., K. J. Rutherford, J. Prasad, and P. K. Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Br. J. Nutr.* 83:167-176.
- Gilliland, S. E. 1979. Beneficial interrelationships between certain microorganisms and humans: candidate microorganisms for use as dietary adjuncts. *J. Food Prot.* 42:164-167.
- Gilliland, S. E., and M. L. Speck. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. *J. Food Prot.* 40:820-823.
- Haenen, D., J. Zhang, C. Souza da Silva, G. Bosch, I. M. Van der Meer, J. Van Arkel, J. J. G. C. Van den Borne, O. Pérez Gutiérrez, H. Smidt, B. Kemp, M. Müller, and G. J. E. J. Hooiveld. 2013. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *The J. Nutr.* 143:274-283.
- Hamaoka, T., and D. H. Katz. 1973. Cellular site of action of various adjuvants in antibody responses to hapten-carrier conjugates. *J. Immunol.* 111:1554-1563.
- Hapfelmeier, S., M. A. E. Lawson, E. Slack, J. K. Kirundi, M. Stoel, M. Heikenwalder, J. Cahenzli, Y. Velykoredko, M. L. Balmer, K. Endt, M. B. Geuking, R. Curtiss, K. D. McCoy, and A. J. Macpherson. 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* 328:1705-1709.
- Hauser, W. E., and J. S. Remington. 1982. Effect of antibiotics on the immune response. *Am. J. Med.* 72:711-716.
- He, B., W. Xu, P. A. Santini, A. D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D. M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. *Immunity* 26:812-826.

- Hengstes, D. J., A. J. Stein, S. W. Casey, and J. U. Que. 1985. Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: influence of antibiotics on colonization resistance. *Infect. Immun.* 47:118-122.
- Hill, D. A., and D. Artis. 2010. Intestinal bacteria and the regulation of immune cell homeostasis. *Annu. Rev. Immunol.* 28:623-667.
- Hooper, L. V., D. R. Littman, and A. J. Macpherson. 2012. Interactions between the microbiota and the immune system. *Science* 336:1268-1273.
- Ivanov, I. I., R. de Llanos Frutos, N. Manel, K. Yoshinaga, D. B. Rifkin, R. Balfour Sartor, B. Brett Finlay, and D. R. Littman. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4:337-349.
- Jakobsson, H. E., C. Jernberg, A. F. Andersson, M. Sjölund-Karlsson, J. K. Jansson, and L. Engstrand. 2010. Short-term antibiotic treatment has differing long-term impact on the human throat and gut microbiome. *PLoS ONE* 5:e9836.
- Janczyk, P., R. Pieper, W. B. Souffrant, D. Bimczok, H.-J. Rothkötter, and H. Smidt. 2007. Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. *ISME J* 1:180-183.
- Jernberg, C., S. Löfmark, C. Edlund, and J. K. Jansson. 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J* 1:56-66.
- Kawase, M., F. He, A. Kubota, K. Yoda, K. Miyazawa, and M. Hiramatsu. 2012. Heat-killed *Lactobacillus gasseri* TMC0356 protects mice against influenza virus infection by stimulating gut and respiratory immune responses. *FEMS Immunol. Med. Microbiol.* 64:280-288.
- Khalifeh, M. S., M. M. Amawi, E. A. Abu-Basha, and I. Bani Yonis. 2009. Assessment of humoral and cellular-mediated immune response in chickens treated with tilmicosin, florfenicol, or enrofloxacin at the time of Newcastle disease vaccination. *Poult. Sci.* 88:2118-2124.
- Lopetuso, L. R., F. Scaldaferri, V. Petito, and A. Gasbarrini. 2013. Commensal clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog* 5:23.
- Luecke, D. H., and L. R. Sibal. 1962. Enhancement by endotoxin of the primary antibody response to bovine serum albumin in chickens. *J. Immunol.* 89:539-544.
- Mazmanian, S. K., C. H. Liu, A. O. Tzianabos, and D. L. Kasper. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122:107-118.
- Merritt, K., and A. G. Johnson. 1963. Studies on the adjuvant action of bacterial endotoxins on antibody formation, V. The influence of endotoxin and 5-fluoro-2-deoxyuridine

- on the primary antibody response of the BALB mouse to a purified protein antigen. *J. Immunol.* 91:266-272.
- Mohamadzadeh, M., S. Olson, W. V. Kalina, G. Ruthel, G. L. Demmin, K. L. Warfield, S. Bavari, and T. R. Klaenhammer. 2005. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc. Natl. Acad. Sci. U.S.A.* 102:2880-2885.
- Moreau, M. C., R. Ducluzeau, D. Guy-Grand, and M. C. Muller. 1978. Increase in the Population of Duodenal Immunoglobulin A Plasmocytes in Axenic Mice Associated with Different Living or Dead Bacterial Strains of Intestinal Origin. *Infect. Immun.* 21:532-539.
- Murai, A., K. Kitahara, S. Okumura, M. Kobayashi, and F. Horio. 2015. Oral antibiotics enhance antibody responses to keyhole limpet hemocyanin in orally but not muscularly immunized chickens. *Anim. Sci. J.* 87:1-9.
- Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* 286:2156-2159.
- Olszak, T., D. An, S. Zeissig, M. Pinilla Vera, J. Richter, A. Franke, J. N. Glickman, R. Siebert, R. M. Baron, D. L. Kasper, and R. S. Blumberg. 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336:489-493.
- Osman, N., D. Adawi, S. Ahrne, B. Jeppsson, and G. Molin. 2004. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of *Lactobacillus* and *Bifidobacterium*. *Dig. Dis. Sci.* 49:320-327.
- Parmentier, H. K., A. L. Klompen, G. De Vries Reilingh, and A. Lammers. 2008. Effect of concurrent intratracheal lipopolysaccharide and human serum albumin challenge on primary and secondary antibody response in poultry. *Vaccine* 26:5510-5520.
- Pathmakanthan, S., C. K. F. Li, J. Cowie, and C. J. Hawkey. 2004. *Lactobacillus plantarum* 299: beneficial in vitro immunomodulation in cells extracted from inflamed human colon. *J. Gastroenterol. Hepatol.* 19:166-173.
- Persson, U. 1977. Lipopolysaccharide-induced suppression of the primary immune response to a thymus-dependent antigen. *J. Immunol.* 118:789-796.
- Petrof, E. O., E. C. Claud, J. Sun, T. Abramova, Y. Guo, T. S. Waypa, S.-M. He, Y. Nakagawa, and E. B. Chang. 2009. Bacteria-free solution derived from *Lactobacillus plantarum* inhibits multiple NF-KappaB pathways and inhibits proteasome function. *Inflamm. Bowel Dis.* 15:1537-1547.
- Rajilić-Stojanović, M., H. G. H. J. Heilig, D. Molenaar, K. Kajander, A. Surakka, H. Smidt, and W. M. De Vos. 2009. Development and application of the human intestinal

- tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11:1736-1751.
- Reikvam, D. H., A. Erofeev, A. Sandvik, V. Grcic, F. L. Jahnsen, P. Gaustad, K. D. McCoy, A. J. Macpherson, L. A. Meza-Zepeda, and F.-E. Johansen. 2011. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PLoS ONE* 6:1-13.
- Renz, H., P. Brandtzaeg, and M. Hornef. 2012. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nat. Rev. Immunol.* 12:9-23.
- Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9:313-323.
- Russell, S. L., M. J. Gold, M. Hartmann, B. P. Willing, L. Thorson, M. Wlodarska, N. Gill, M.-R. Blanchet, W. W. Mohn, K. M. McNagny, and B. B. Finlay. 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep.* 13:440-447.
- Salonen, A., J. Nikkilä, J. Jalanka-Tuovinen, O. Immonen, M. Rajilić-Stojanović, R. A. Kekkonen, A. Palva, and W. M. de Vos. 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. *J. Microbiol. Methods* 81:127-134.
- Schokker, D., J. Zhang, S. A. Vastenhouw, H. G. H. J. Heilig, H. Smidt, J. M. J. Rebel, and M. A. Smits. 2015. Long-lasting effects of early-life antibiotic treatment and routine animal handling on gut microbiota composition and immune system in pigs. *PLoS ONE* 10:e0116523.
- Schultz, M., C. Veltkamp, L. A. Dieleman, W. B. Grenther, P. B. Wyrick, L. Tonkonogy, and R. B. Sartor. 2002. *Lactobacillus plantarum* 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm. Bowel Dis.* 8:71-80.
- Sekirov, I., N. M. Tam, M. Jogova, M. L. Robertson, Y. Li, C. Lupp, and B. Brett Finlay. 2008. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect. Immun.* 76:4726-4736.
- Shanmugasundaram, R., and R. K. Selvaraj. 2012. In vivo-lipopolysaccharide injection alters CD4<sup>+</sup>CD25<sup>+</sup> cell properties in chickens. *J. Anim. Sci.* 90:2498-2504.
- Simon, K., G. De Vries Reilingh, B. Kemp, and A. Lammers. 2014. Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers. *Poult. Sci.* 93:1-11.

- Smith, K., K. D. McCoy, and A. J. Macpherson. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* 19:59-69.
- Stecher, B., and W.-D. Hardt. 2011. Mechanisms controlling pathogen colonization of the gut. *Curr. Opin. Microbiol.* 14:82-91.
- Sullivan, Å., and C. E. Nord. 2001. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis* 1:101-114.
- Van Der Waaij, D., J. M. Berghuis-de Vries, and J. E. C. Lekkerkerk-van der Wees. 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J Hyg (Lond)* 69:405-411.
- Van Kaer, L., V. V. Parekh, and L. Wu. 2011. Invariant natural killer T cells: bridging innate and adaptive immunity. *Cell Tissue Res.* 343:43-55.
- Willing, B. P., S. L. Russell, and B. Brett Finlay. 2011. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat. Rev. Microbiol.* 9:233-243.
- Woo, P. C. Y., H.-W. Tsoi, L.-P. Wong, H. C. H. Leung, and K.-Y. Yuen. 1999. Antibiotics modulate vaccine-induced humoral immune response. *Clin. Diagn. Lab. Immunol.* 6:832-837.

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## **CHAPTER 5**

### **Effects of early life dextran sulfate sodium administration on pathology and immune response in broilers and layers**

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## ABSTRACT

Intestinal pathology early in life may affect immune development and therefore immune responses later in life. Dextran sulfate sodium (**DSS**) induces colitis in rodents and is a widely used model for inflammatory bowel diseases. The present study investigated DSS as a model for early life intestinal pathology and its consequences on intestinal pathology, ileal cytokine and immunoglobulin mRNA expression levels as well as the antibody response towards an immunological challenge later in life in chickens. Broiler and layer chicks received 2.5 % DSS in drinking water during d 11 through d 18 post hatch or plain drinking water as a control. As an immunological challenge all birds received a combination of *Escherichia coli* lipopolysaccharide (**LPS**) and Human Serum Albumin (**HuSA**) intramuscularly (**i.m.**) at d 35 and antibody titers against LPS, HuSA, and keyhole limpet hemocyanin (**KLH**) were determined to investigate effects of intestinal inflammation early in life on humoral immunity later in life. DSS treated birds showed a decrease in BW from which broilers quickly recovered, but which persisted for several weeks in layers. Histological examination of intestinal samples showed symptoms similar to those in rodents, including shortening and loss of villi and crypts as well as damage of the epithelial cell layer of different parts of the intestine. Effects of DSS on intestinal morphology were less severe in broilers that also showed a lower mortality in response to DSS than layers. No effect of DSS on ileal cytokine expression levels could be observed, but ileal immunoglobulin expression levels were decreased in DSS treated broilers that also showed lower antibody titers against LPS in response to the challenge. In conclusion, DSS may serve as a model for intestinal pathology early in life, although more research on the appropriate dose is necessary and is likely to differ between breeds. Results from the present study could indicate that broilers are less susceptible to DSS compared with layers or have a better capacity to recover from intestinal pathology.

**Key words:** chicken, DSS, intestinal homeostasis, immune response

## INTRODUCTION

Early life intestinal pathology may have profound effects on immune development and immune responses later in life, due to an interaction between intestinal microbiota and the host immune system (Round and Mazmanian, 2009; Hill and Artis, 2010; Hooper et al., 2012). It has been shown that colitis, a recurring problem in practice caused by bacterial pathogens, also affects mucosal functions of the ileum (Amit-Romach et al., 2006), which is considered the site of immune activation. Furthermore colitis leads to enhanced ileal expression of pro-inflammatory cytokines in rats (Barada et al., 2006). Administration of dextran sulfate sodium (**DSS**) may serve as a model for intestinal pathology. DSS reliably induces colitis in rodents when administered via drinking water for several days and is therefore widely used in models for inflammatory bowel disease. The exact working mechanism by which DSS induces colitis is not entirely clear, but so far it has been suggested that DSS compromises the intestinal function in different ways. Johansson et al. (2010) have shown that DSS alters the thickness of the mucus layer and makes it permeable to bacteria. After 12 h of DSS exposure bacteria were shown to be in contact with the epithelial cell layer, where bacteria can elicit an inflammatory response. DSS also decreases the capacity of macrophages to phagocytose bacteria, making it possible for bacteria to invade the lamina propria (Ohkusa et al., 1995; Stevceva et al., 2001). It seems that the malfunction of macrophages plays an important role in the initial acute phase of DSS induced colitis, which also develops in SCID mice lacking B and T cells (Dieleman et al., 1994). Furthermore, Laroui et al. (2012) suggested that DSS disrupts the intestinal barrier by linking to medium chain fatty acids and forming vesicles. These vesicles fuse with epithelial cells of the colon and consequently affect major cell pathways, leading to a disruption of the intestinal barrier and an induction of inflammatory responses. Inflammatory responses include mucosal neutrophil infiltration and an upregulation pro-inflammatory cytokines (Yan et al., 2009; Dutra et al., 2011).

Apart from inflammation of the colon, DSS treated animals also show changes in microbiota composition (Okayasu et al., 1990; Faure et al., 2006; Lupp et al., 2007). The influence of intestinal microbiota on the development of various parts of the host immune system and the maintenance of immune homeostasis has been summarized in several reviews (Round and Mazmanian, 2009; Hill and Artis,

2010; Hooper et al., 2012). Due to changes in intestinal microbiota composition and stimulation of inflammatory responses, administration of DSS early in life when the immune system is not fully mature yet may therefore affect host immune development and consequently immune responses later in life. Given its high reproducibility and easy administration, DSS induced colitis might serve as a model for the consequences of early life intestinal pathology on immune development and long-term immune responses in chickens.

The present study therefore aimed to investigate the possibility to use DSS administration as a model for early life intestinal pathology in chickens. Effects of DSS administration early in life on the development of ileal cytokine expression levels as well as the immune response towards a systemic immune challenge later in life were investigated. Although DSS primarily induces inflammation of the colon and not the ileum, effects on the ileum where immune maturation is initiated are likely. Effects of DSS on immune development and immune response in broilers as well as layers was studied, since an earlier study indicated differences in immune development between the two breeds in that broilers and layers seem to have different immune strategies (Simon et al., 2014).

## **MATERIALS AND METHODS**

### ***Chickens and Housing***

The present study was conducted with Ross 308 broiler hens (n=130) and Lohman Brown Classic laying hens (n=130). Fertilized eggs from both breeds were obtained from 2 commercial hatcheries (Lagerwey Hatchery, Lunteren, The Netherlands and Ter Heerdt Hatchery, Zevenaar, The Netherlands) and were incubated at our department under standard incubation conditions appropriate for each breed. From embryonic d 19 onwards eggs were checked every 3 h for signs of hatching. Every 3 h hatched chicks from both breeds were removed from the incubator and randomly assigned to one of 2 treatments: control or DSS treated. All birds received a standard commercial diet appropriate for each breed containing a coccidiostat (salinomycin). Feed and water was accessible ad libitum. Control birds received plain drinking water, DSS birds received 2.5 % DSS (Sigma-Aldrich

Chemie GmbH, Steinheim, Germany, catalog 42867-100G) in drinking water from d 10 to d 18 post hatch (**p.h.**). Water containing DSS was exchanged every day. Birds were housed per breed in separate rooms due to different temperature requirements of the two breeds. Birds were floor-housed in groups in pens of 1 x 2 m containing wood shavings with a regimen of 16L:8D. Birds were not vaccinated.

The dose of DSS was based on results of a pilot study. In the pilot study 3 chicks per breed received either 0.5 %, 1.0 % , 1.5%, 2.0 %, or 2.5 % DSS in drinking water from d 10 to d 18 p.h. Control groups of each breed received plain drinking water throughout the whole experimental period. On d 18 all chicks were sacrificed and colon samples were obtained for histological examination of intestinal damage and signs of inflammation. Pathology of the colon was only observed in some animals of the 2.5 % group, but not in animals that received a lower dose of DSS and 2.5 % DSS was chosen as the dose to be used in the present follow-up study.

On d 35 p.h. 15 birds per treatment received an immunological challenge consisting of i.m. administered *Escherichia coli* lipopolysaccharide (**LPS**) (2.5 mg/kg) (LPS from *E. coli* 055:B5, Sigma-Aldrich Chemie GmbH, Steinheim, Germany, catalog L2880) in combination with Human Serum Albumin (**HuSA**) (0.5 mg/kg) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, catalog A3782).

This study was approved by the Animal Welfare Committee of Wageningen University and Research Centre in accordance with Dutch laws and regulations on the execution of animal experiments.

### ***Measurements and Sampling***

Birds were weighed at hatch and then once a week at the same time of day. Birds that received an LPS/HuSA challenge were additionally weighed the day before and the day of the challenge and each day for the first 7 days after administration of the challenge.

Water and feed intake was recorded daily during DSS administration.

Ileal samples were taken at d 10, 11 (8 h after first administration of DSS), 14, 18, and 35 p.h. Per time point 10 birds per breed and treatment were sacrificed. Ileal midsections of 1 cm between Meckel's diverticulum and ceco-iliac junction were stored overnight at 4°C in RNA later (Life Technologies, Carlsbad, CA,

USA; catalog AM7020). After removal of RNA later, ileal samples were stored at -80°C until analysis.

Length of colon and ceca was measured on d 18 and 35 after careful removal of colonic and cecal contents.

Ileal, colonic, and cecal samples were collected for histology from 3 birds per breed per treatment at d 18 p.h. in order to visualize pathological effects of DSS. Swiss rolls of intestinal samples were immediately fixed overnight in Carnoy's fixative consisting of 60% methanol, 30% chloroform, and 10% glacial acetic acid. After embedding in paraffin, sections of 7 µm were cut and stained with Hematoxylin and Eosin (**HE**).

Blood was collected from the wing vein of birds that received an LPS/HuSA challenge on the day before administration of the challenge and on d 3, 7, and 14 after administration of the challenge. Antibody titers were determined by ELISA as described previously (Simon et al., 2014), titer calculations were performed as described by De Koning et al. (2015).

### ***RNA Isolation and cDNA Synthesis***

Total RNA was extracted from ileal midsections using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA; catalog 15596-026) according to the manufacturer's recommendations. RNA concentrations were measured by a spectrophotometer (Multiscan GO, Thermo Fisher Scientific, Vantaa, Finland) and 1 µL was analyzed on a 1% agarose gel to check RNA integrity.

Prior to cDNA synthesis, RNA was further purified by an extra precipitation step, since remainders of DSS in tissue samples have been reported to inhibit reverse transcription in mice (Kerr et al., 2012; Viennois et al., 2013). Inhibition of reverse transcription was also observed in samples of the present study. Samples were therefore purified with lithium chloride (**LiCl**), using a protocol adapted from Viennois et al. (2013). A deviation from the protocol of Viennois et al. (2013) was that samples in our protocol were incubated with 0.5 instead of 0.1 volumes of LiCl. After the final removal of supernatant the pellet was dissolved in 20 µL diethylpyrocarbonate (**DEPC**)-treated water. RNA concentrations were equaled to 500 ng/µl by dilution in DEPC-treated water after measuring RNA concentrations on a spectrophotometer.

After LiCl purification RNA samples were DNase treated (all chemicals for DNA synthesis originated from Invitrogen, Carlsbad, CA, USA). 1  $\mu$ L DNase I and 1  $\mu$ L DNase I reaction buffer (DNase I amplification grade kit, also containing 25 mM EDTA; catalog 18068-015) were added to 500 ng of total RNA and incubated for 15 min at room temperature at a total volume of 12  $\mu$ L. 1  $\mu$ L 25 mM EDTA was added and samples were incubated at 65°C for 10 min to inactivate DNase I. After incubation samples were quickly cooled down on ice.

For cDNA synthesis 300 ng random primers (cat 48190-011), 1  $\mu$ L 10mM dNTPs (catalog 18427-013), and 0.9  $\mu$ L DEPC-treated water were added and samples were incubated at 70°C for 10 min. After quickly cooling the samples down on ice samples were incubated with 1  $\mu$ L RNase OUT (catalog 10777-019), 1  $\mu$ L Superscript II, 2  $\mu$ L 0.1 M DTT, and 4  $\mu$ L 5x First Strand Buffer (SuperScript II Reverse Transcriptase kit, catalog 18064-014) at 37°C for 50 min and subsequently at 70°C for 10 min. After incubation samples were quickly cooled down on ice and stored at -20°C until further use.

### ***Real-Time Quantitative PCR***

Real-time quantitative PCR was performed relative mRNA expression levels of pro-inflammatory cytokines IL-1 $\beta$ , IL-12p40, and interferon gamma (**IFN $\gamma$** ), anti-inflammatory cytokine IL-10, as well the immunoglobulins IgM, IgY, and IgA. Additionally relative mRNA expression levels of B cell activating factor (**BAFF**) were investigated, which is a member of the TNF cytokine family and plays a role in the regulation of inflammatory responses. The 28S rRNA was used as a reference gene. Primer sequences are listed in Table 1. Samples of cDNA were diluted 1:5,000 for 28S and 1:50 for target genes. A reaction volume of 25  $\mu$ L per well was reached by adding 5  $\mu$ L cDNA, 12.5  $\mu$ L SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK; catalog 4309155), 1.25  $\mu$ L 10  $\mu$ M forward and reverse primer, 0.31  $\mu$ L VisiBlue inert coloring dye (TATAA Biocenter, Gothenburg, Sweden), and 5.94  $\mu$ L endotoxin-free water (G-Biosciences, St. Louis, MO, USA; cat 786-670).

Real-time quantitative PCR was performed on a Bio-Rad MyiQ real-time PCR detection system with the following protocol: 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 59°C, and 36 s at 72°C. Melting curves were

**Table 1.** Primer sequences

Gene	Accession number	Primer sequence <sup>1</sup> (5' → 3')
<b>28S</b>	DQ018756	F: GGC-GAA-GCC-AGA-GGA-AAC-T R: GAC-GAC-CGA-TTT-GCA-CGT-C
<b>IL-1β</b>	AJ245728	F: CAG-CAG-CCT-CAG-CGA-AGA-G R: CTG-TGG-TGT-GCT-CAG-AAT-CCA
<b>IL-10</b>	AJ621614	F: CGC-TGT-CAC-CGC-TTC-TTC-A R: TCC-CGT-TCT-CAT-CCA-TCT-TCT-C
<b>IL-12p40</b>	NM_213571.1	F: GAC-CCA-CGA-GAT-TAT-CAG-CTA-CAG-T R: TGC-TTG-GCT-CTT-TAT-AGC-TTT-TCA
<b>INF-γ</b>	Y07922	F: GTG-AAG-AAG-GTG-AAA-GAT-ATC-ATG-GA R: GCT-TTG-CGC-TGG-ATT-CTC-A
<b>BAFF</b>	AY263378.1	F: CAT-GCA-ATC-ACC-CTG-AAA-AC R: TTC-TCC-CCC-TGT-TTC-TGT-TC
<b>IgM</b>	X01613.1	F: GCA-TCA-GCG-TCA-CCG-AAA-GC R: TCC-GCA-CTC-CAT-CCT-CTT-GC
<b>IgY</b>	X07174.1	F: ATC-ACG-TCA-AGG-GAT-GCC-CG R: ACC-AGG-CAC-CTC-AGT-TTG-G
<b>IgA</b>	S40610	F: GTC-ACC-GTC-ACC-TGG-ACT-ACA R: ACC-GAT-GGT-CTC-CTT-CAC-ATC

<sup>1</sup>F = forward; R = reverse

obtained after each run by detection of fluorescence at 1°C intervals from 60°C to 90°C. Contamination of samples with genomic DNA was checked with the ValidPrime Vertebrate kit (TATAA Biocenter, Gothenburg, Sweden) according to the manufacturer's recommendations. Relative expression ratios of target genes were calculated as follows:  $\text{ratio} = \frac{E^{Ct_{28S}}}{E^{Ct_{\text{target gene}}}}$

The cut-off value was set at a  $C_t$  of 30. Samples above a  $C_t$  of 30 were classified as undetermined and were allocated an arbitrary  $C_t$  of 40 for calculations of the relative gene expression ratio.

### ***Statistical Analysis***

Data were analyzed using a mixed linear model in SAS 9.2 (SAS Institute Inc., Cary, NC). Since broilers and layers were kept in two different rooms, breed is confounded with room and therefore data was analyzed separately per breed. Differences and similarities between breeds are therefore discussed in a descriptive way.

Data on BW, growth in response to the administered LPS/HuSA challenge, natural and specific antibody titers were obtained from the same animals on several time points and cannot be seen as independent. Therefore a repeated measures analysis was incorporated in the statistical model. Pen was considered the experimental unit and was included as a random effect in the model. Data on feed and water intake during DSS administration were recorded daily on pen level. Data on feed and water intake can therefore not be seen as independent and a repeated measures analysis was incorporated in the statistical model.

Data on colon and cecum length, and data on relative ileal cytokine and immunoglobulin mRNA expression levels was obtained from different animals at each time point and variance increased in time. Data were therefore analyzed per time point. Pen was considered the experimental unit and was included as a random effect in the model. A natural log transformation was performed on data on relative ileal cytokine and immunoglobulin mRNA expression levels to approximate a normal distribution. For time points at which ileal cytokine expression levels remained undetermined or were determined in less than 10% of the birds, no statistical analysis was performed.

Outlier analysis including influence diagnostics was performed. When an outlier was detected, both results before and after removal of the outlier from the dataset are presented.

Normality was checked on the residuals. Data are displayed as means and corresponding SE. Differences are considered significant where  $P \leq 0.05$ .

## RESULTS

### *Mortality*

None of the control birds dropped out of the experiment, but a number of DSS treated birds of both breeds either died or were removed from the experiment due to their poor condition. In total two DSS treated broilers (3.1 %) and seven DSS treated layers (10.8 %) either died or had to be removed from the experiment. The two broilers were found dead after 3 d of DSS treatment. In DSS treated layers one bird was removed after 4 d, one bird after 6 d, and two birds after 7 d of DSS treatment. Furthermore, one bird was found dead 1 d after cessation of DSS administration, and one bird was removed 3 d after cessation of DSS administration and another bird 9 d after cessation of DSS administration.

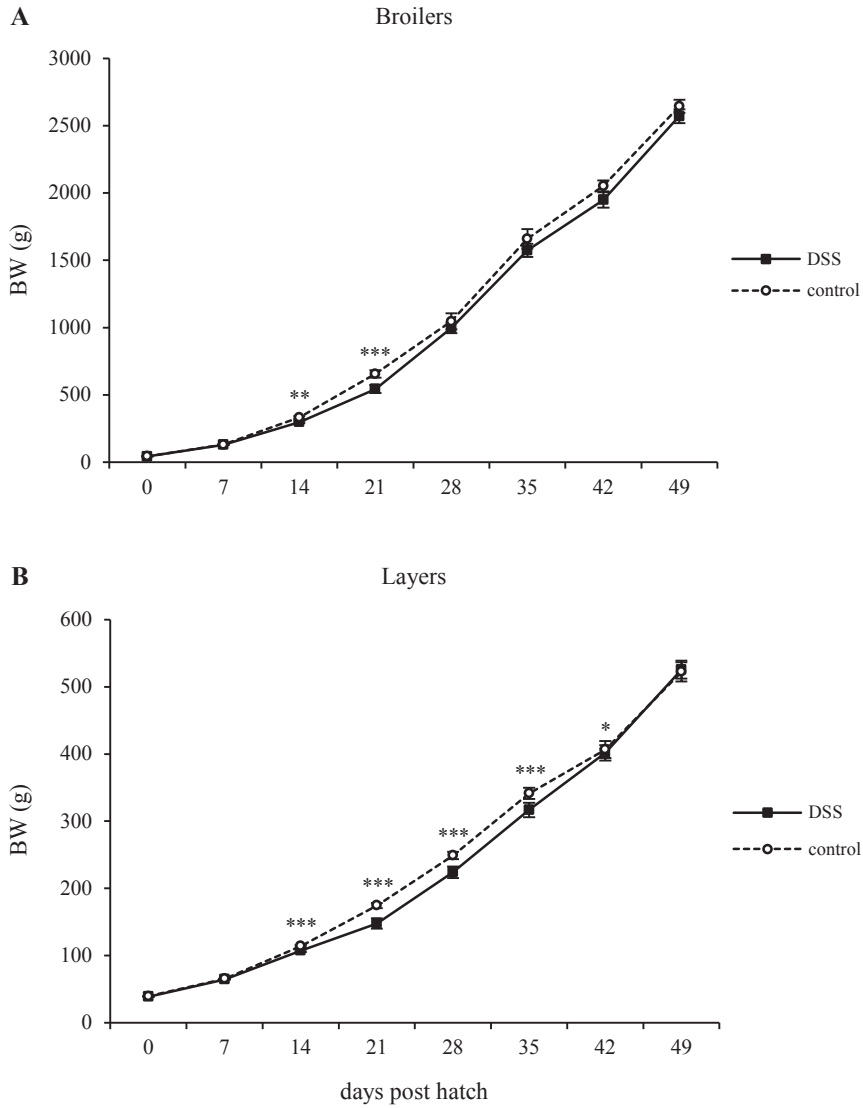
### *BW*

In broilers as well as in layers BW was influenced by a combination of treatment and time ( $P = 0.014$  and  $P < 0.001$ , respectively). Compared with control broilers, BW of DSS treated broilers was lower on d 14 ( $P = 0.011$ ) and d 21 ( $P < 0.001$ ), but had recovered by d 28 (Figure 1A). Compared with broilers BW of DSS treated layers was affected for a longer period of time. Compared with control layers, DSS treated layers showed lower BW from d 14 through d 42 ( $P < 0.001$  for d 14 through d 35,  $P = 0.01$  for d 42) (Figure 1B).

### *Water Intake During DSS Treatment*

In broilers DSS treatment affected water intake during the period of DSS administration. During DSS administration pens of DSS treated broilers had a lower daily water intake ( $314.6 \pm 9.2$  ml) compared with pens of control birds ( $360.7 \pm 5.3$  ml) ( $P = 0.005$ ).

In layers water intake during DSS treatment was affected by a combination of treatment and time ( $P = 0.009$ ). On d 2 of DSS administration pens of DSS treated layers had a higher water intake ( $150.1 \pm 18.8$  ml) compared with pens of control birds ( $124.1 \pm 3.7$  ml) ( $P = 0.02$ ). However, by d 7 of DSS administration the pens of DSS treated layers had a lower water intake ( $93.2 \pm 5.9$  ml) compared with control birds ( $116.4 \pm 3.2$  ml) ( $P = 0.04$ ).



**Figure 1.** BW of DSS treated and control broilers (A) and layers (B). DSS treated birds received 2.5 % DSS in drinking water from d 11 through d 18. Data are displayed as means and corresponding SE. Significant differences between treatment groups are indicated by asterisks (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ ).

***Feed Intake During DSS Treatment***

In broilers and layers DSS treatment affected feed intake during the period of DSS administration. During DSS administration, the pens of DSS treated broilers showed a lower daily feed intake ( $338.3 \pm 7.9$  g) compared to control birds ( $425.2 \pm 20.4$  g) ( $P = 0.009$ ). The same was true for pens of DSS treated layers which showed a lower daily feed intake ( $232.8 \pm 16.4$  g) compared with control birds ( $268.7 \pm 16.1$  g) ( $P = 0.02$ ).

***Colon and Cecum Length***

In broilers and layers both colon and cecum length were affected by DSS treatment. On d 18, i.e. the end of DSS administration, DSS treated broilers had a shorter colon ( $4.5 \pm 0.3$  cm) and cecum ( $6.7 \pm 0.2$  cm) compared with control birds ( $5.0 \pm 0.3$  cm and  $10.7 \pm 0.3$ , respectively) ( $P = 0.01$  and  $P < 0.001$ , respectively). On d 35 colon and cecum length between DSS treated (colon  $7.3 \pm 0.4$  cm; cecum  $12.8 \pm 0.8$  cm) and control broilers (colon  $7.6 \pm 0.7$  cm; cecum  $14.6 \pm 0.7$  cm) did not differ any more. Like broilers, DSS treated layers had a shorter colon ( $3.7 \pm 0.2$  cm) and cecum ( $4.4 \pm 0.2$  cm) compared with control birds ( $4.6 \pm 0.2$  cm and  $7.1 \pm 0.2$  cm, respectively) ( $P = 0.009$  and  $P < 0.001$ , respectively) on d 18. Colons of DSS treated layers ( $4.4 \pm 0.5$  cm) still tended to be shorter on d 35 compared with control birds ( $5.6 \pm 0.2$  cm) ( $P = 0.07$ ). Cecum length of DSS treated ( $7.1 \pm 0.9$  cm) and control layers ( $8.8 \pm 0.3$  cm) did not differ on d 35.

***Histological Observations***

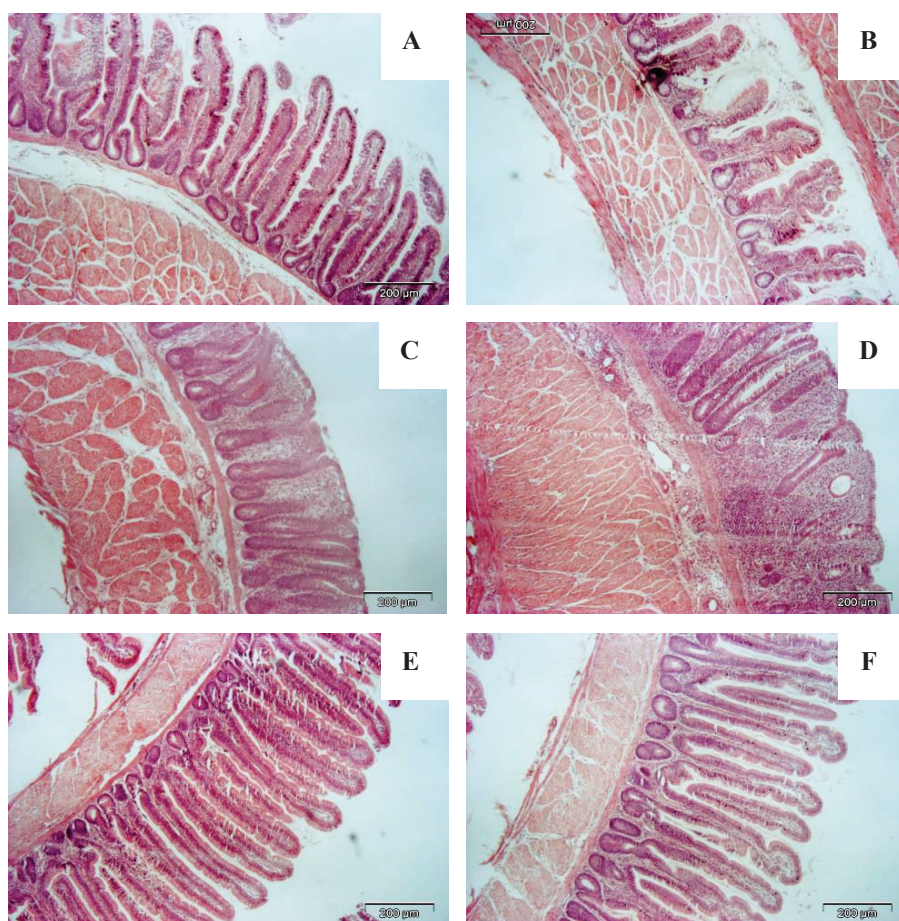
Histological observations on sections of colon, cecum, and ileum of both breeds were carried out for 3 birds per breed per treatment. All three DSS treated broilers showed a shortening and thickening of colonic villi and in some parts a disruption of the epithelium. Ceca of all three DSS treated broilers partly showed a loss of crypts. In ileum of broilers no apparent effects of DSS treatment could be observed.

All three DSS treated layers showed a shortening and thickening of colonic villi with a disruption of the epithelial cell layer and an almost complete loss of crypts in the ceca. Observations in the ileum were variable with a loss of villi,

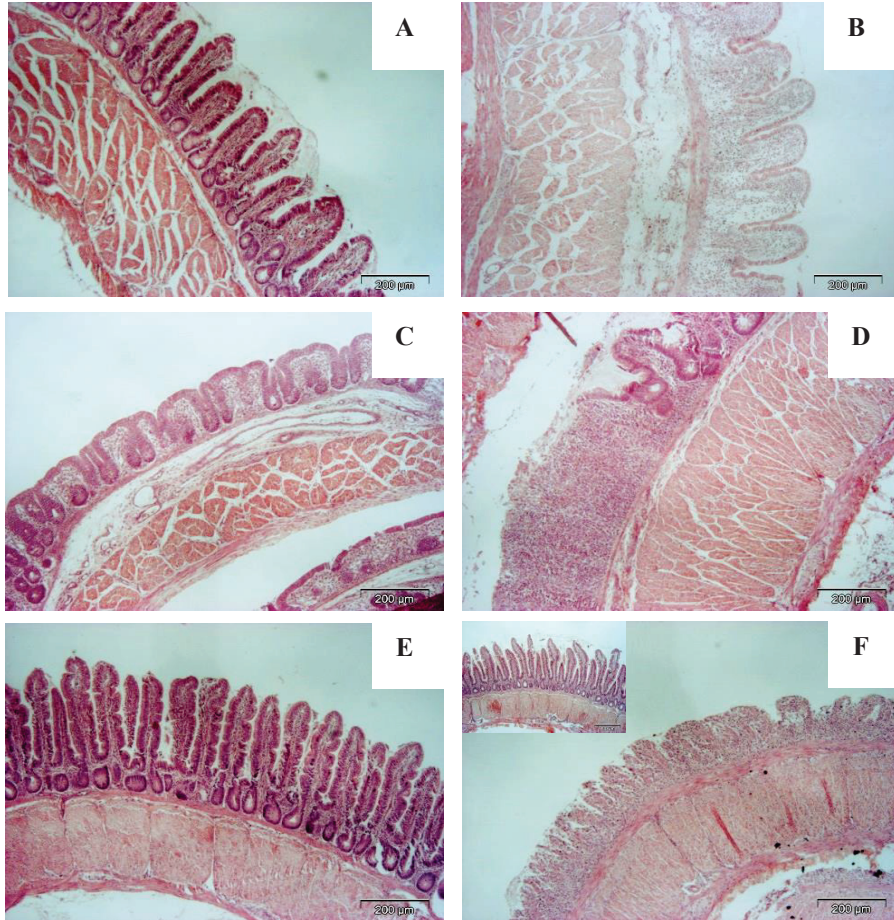
crypts and the epithelial cell layer in one bird, while the two other birds showed an ileum similar to control birds.

In both colon and cecum of broilers and layers infiltration of blood cells was observed which was in accordance with the macroscopic observation of intestinal bleeding in these tissues during dissection.

Intestinal damage of broilers (Figure 2A through 2F) seemed less severe compared with layers (Figure 3A through 3F).



**Figure 2.** HE stained sections of different parts of the intestine of broilers. A: colon of control broiler, B: colon of DSS treated broiler, C: cecum of control broiler, D: cecum of DSS treated broiler, E: ileum of control broiler, F: ileum of DSS treated broiler.



**Figure 3.** HE stained sections of different parts of the intestine of layers. A: colon of control layer, B: colon of DSS treated layer, C: cecum of control layer, D: cecum of DSS treated layer, E: ileum of control layer, F: ileum of DSS treated layer. The insertion in F shows the ileum of another DSS treated layer showing less damage.

### ***Relative BAFF and Immunoglobulin mRNA Expression Levels***

Figures 4A through 4F display relative BAFF and immunoglobulin expression levels during administration of DSS.

In broilers BAFF expression levels did not differ between DSS treated and control broilers at any time point (Figure 4A). IgM expression levels tended to be

lower in DSS treated broilers on d 14 ( $P = 0.065$ ) and were lower on d 18 ( $P = 0.05$ ) compared with control broilers (Figure 4B). IgY expression levels tended to be lower in DSS treated broilers compared with control broilers on d 18 ( $P = 0.09$ ) (Figure 4C). IgA expression levels were lower in DSS treated birds on d 14 ( $P = 0.05$ ) and d 18 ( $P = 0.024$ ) compared with control birds (Figure 4D).

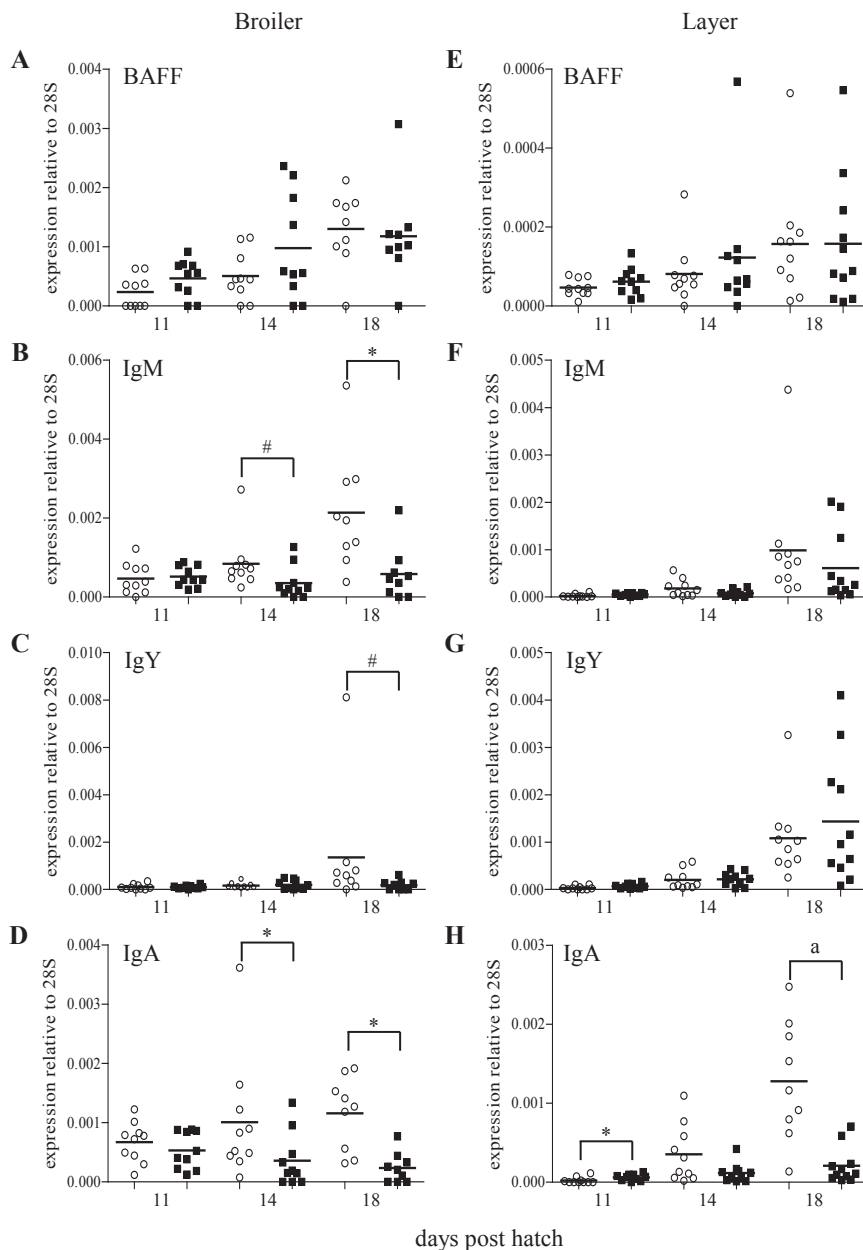
In layers BAFF expression levels did not differ between DSS treated and control layers at any time point (Figure 4E). IgM and IgY expression levels did not differ between DSS treated and control birds at any time point (Figure 4F and 4G). IgA expression levels were higher in DSS treated layers compared with control layers on d 11 ( $P = 0.034$ ) (Figure 4H). At d 18 IgA expression levels did not differ between DSS treated and control birds, but when an outlier (detected by the influence diagnostics in SAS 9.2) was removed from the dataset, DSS treated birds had lower IgA expression levels compared with control birds on that day ( $P = 0.002$ ).

### ***Relative Ileal Cytokine mRNA Expression Levels***

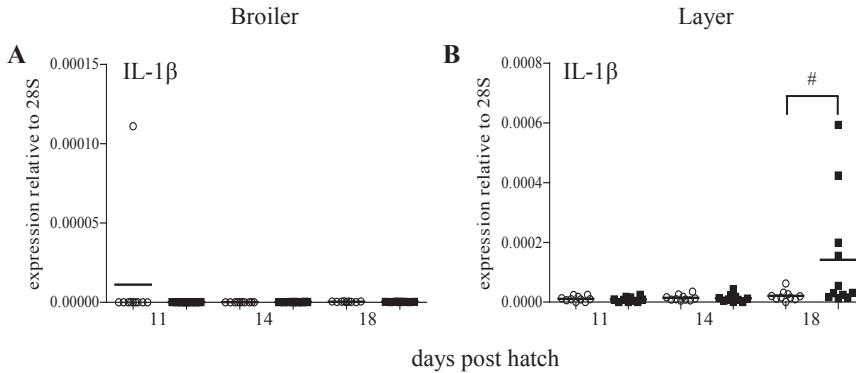
Figures 5A and 5B display relative IL-1 $\beta$  expression levels during administration of DSS. Data of other pro- or anti-inflammatory cytokines measured is not shown.

In broilers IL-1 $\beta$  (Figure 5A), IL-12p40, and IL-10 expression levels remained undetermined or were determined in less than 10% of the birds at all time points. IFN $\gamma$  expression levels remained undetermined from d 10 through d 18 and did not differ between DSS treated and control broilers at d 35.

In layers IL-1 $\beta$  expression levels tended to be higher in DSS treated layers compared with control layers on d 18 ( $P = 0.064$ ) (Figure 5B), but did not differ from expression levels of control layers at any other time point. IL-12p40 expression levels remained undetermined or were determined in less than 10% of the layers at all time points. IFN $\gamma$  expression levels remained undetermined or were determined in less than 10% of the layers from d 10 through d 14 and did not differ between DSS treated and control layers at d 18 and d 35. IL-10 expression levels remained undetermined or were determined in less than 10% of the layers from d 10 through d 18 and did not differ between DSS treated and control layers at d 35.



**Figure 4.** Ileal BAFF and immunoglobulin mRNA expression levels of broilers and layers during administration of DSS. Treatments were 2.5 % DSS in drinking water from d 11 through d 18 (black squares) and plain drinking water as a control (open circles). Data are displayed as means and corresponding SE. Differences between treatment groups within one day are indicated as follows: \* =  $P \leq 0.05$ , # =  $P \leq 0.1$ , a = significant difference ( $P \leq 0.05$ ) between treatment groups after removal of an outlier.



**Figure 5.** Ileal IL-1 $\beta$  mRNA expression levels of broilers and layers during administration of DSS. Treatments were 2.5 % DSS in drinking water from d 11 through d 18 (black squares) and plain drinking water as a control (open circles). Data are displayed as means and corresponding SE. Differences between treatment groups within one day are indicated as follows: # =  $P \leq 0.1$ .

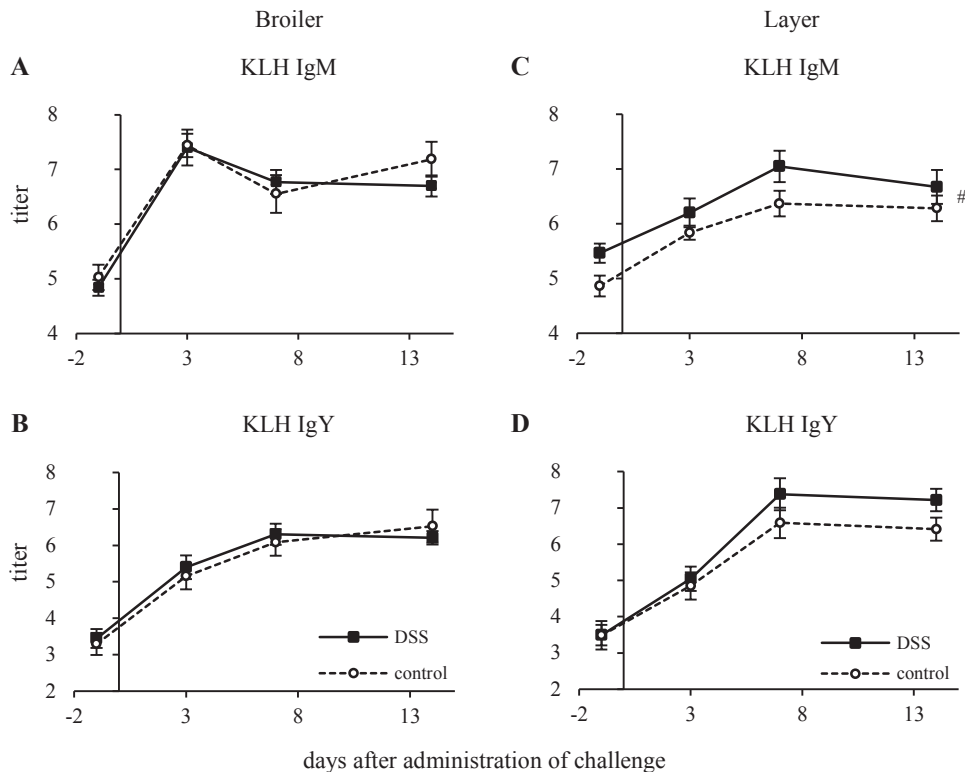
### Weight Loss Around Challenge

In response to the administered LPS/HuSA challenge all birds showed weight loss one day after administration of the challenge. DSS treated and control birds of both breeds did not differ in terms of weight loss. For broilers weight loss was  $105.8 \pm 11.8$  g for DSS treated broilers and  $120.0 \pm 6.3$  g for control broilers. For layers weight loss was  $13.8 \pm 2.1$  g for DSS treated layers and  $16.4 \pm 1.8$  g for control layers.

### Natural Antibody Titers

In broilers NAb titers against KLH (Figure 6A and 6B) were not influenced by DSS treatment.

In layers DSS treated birds tended to have higher IgM titers against KLH ( $6.3 \pm 0.2$ ) compared with control birds ( $5.8 \pm 0.1$ ) ( $P = 0.06$ ) (Figure 6C). IgY titers against KLH were not affected by DSS treatment in layers (Figure 6D).



**Figure 6.** Natural antibody titers against KLH in response to an i.m. LPS/HuSA challenge in broilers and layers. DSS treated birds received 2.5 % DSS in drinking water from d 11 through d 18. Data are displayed as means and corresponding SE. Differences between treatment groups are indicated by a hashtag ( $P \leq 0.1$ , main effect of treatment).

### Specific Antibody Titers

In broilers antibody titers against LPS were influenced by DSS treatment. DSS treated birds tended to have lower IgM titers against LPS ( $5.9 \pm 0.2$ ) compared with control birds ( $6.5 \pm 0.2$ ) ( $P = 0.08$ ) (Figure 7A). Furthermore DSS treated birds had lower IgY titers against LPS ( $2.8 \pm 0.2$ ) compared with control birds ( $3.9 \pm 0.2$ ) ( $P = 0.006$ ) (Figure 7B).

In layers DSS treatment influenced IgM titers against LPS in that DSS treated birds tended to have higher IgM titers against LPS ( $6.9 \pm 0.2$ ) compared with

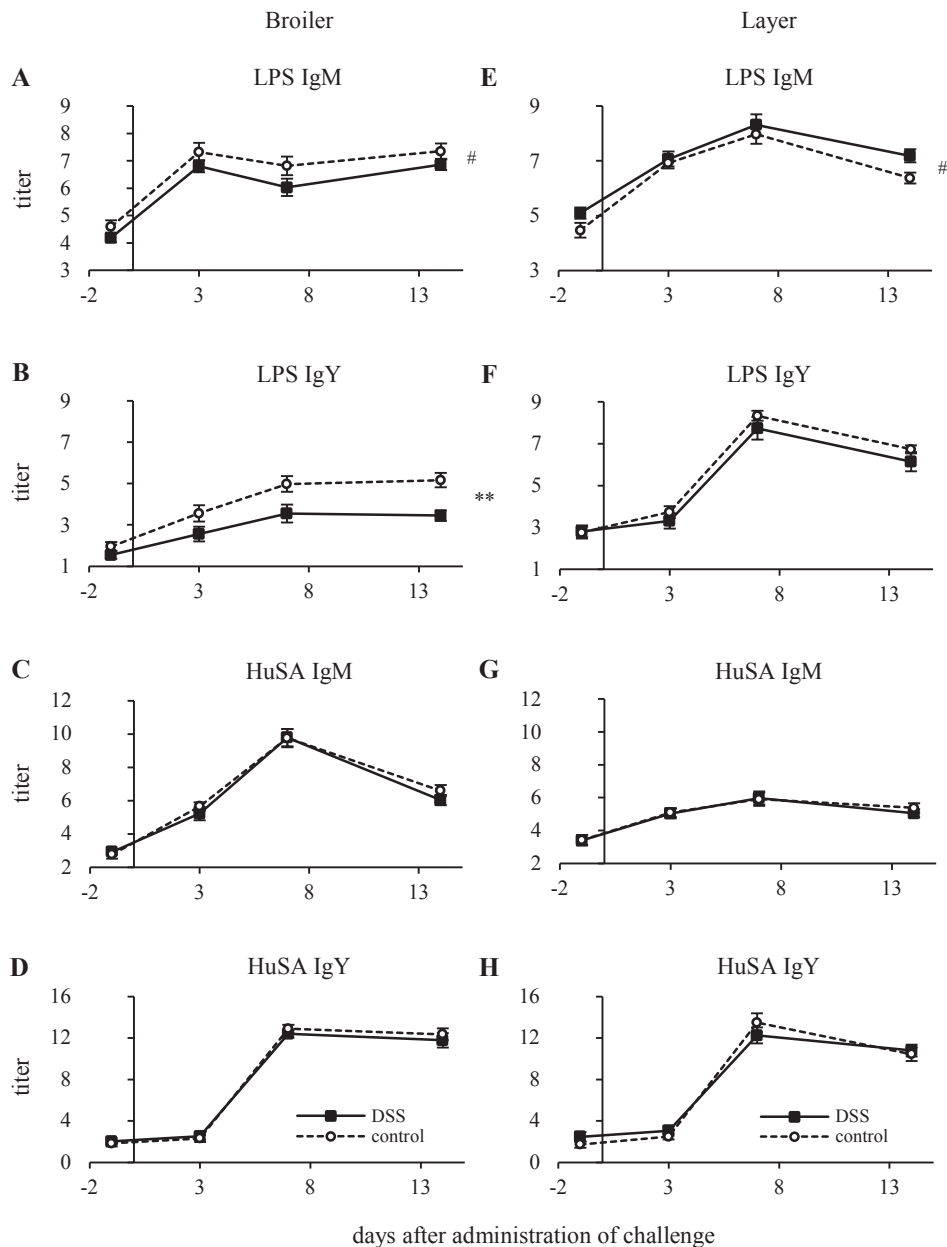
control birds ( $6.4 \pm 0.2$ ) ( $P = 0.08$ ) (Figure 7E). DSS treatment did not influence IgY titers against LPS in layers (Figure 7F).

In both breeds DSS treatment did not influence IgM or IgY titers against HuSA (Figure 7C, 7D, 7G, 7H).

## DISCUSSION

The present study investigated the effects of 2.5% DSS in drinking water early in life of broilers and layers on BW, intestinal morphology, ileal cytokine and Ig mRNA expression levels, as well as natural antibody levels and the specific antibody response towards an LPS/HuSA challenge later in life.

Results of the present study show that apart from rodents, DSS may also be used in chickens as a model for disruption of intestinal homeostasis. Typical symptoms of DSS-induced colitis could be observed, such as bloody feces and BW loss in DSS treated birds. Moreover, DSS treated birds showed alterations in intestinal morphology like degradation of the epithelium up to complete loss of villi and crypts in different parts of the intestine and DSS treated birds also had a shorter colon and ceca. These results are in accordance with findings in rodents, which show weight loss, bloody diarrhea, reduction in colon length and morphological changes in the colon and caecum, such as damage of the epithelium, and shortening or loss of crypts (Cooper et al., 1993; Yan et al., 2009; Dutra et al., 2011; Perše and Cerar, 2012). Findings of the present study are also in line with two very recent studies conducted in broilers, which showed that DSS treated broilers developed symptoms similar to those in rodents, including morphological changes throughout the intestine (Menconi et al., 2015) as well as an impaired intestinal barrier function (Kuttappan et al., 2015) also observed in rodents (Ohkusa et al., 1995; Stevceva et al., 2001; Yan et al., 2009; Johansson et al., 2010; Laroui et al., 2012). Menconi et al. (2015) also reported effects of DSS on the ileum, such as a shortening of villi in DSS treated broilers. In the present study clearly noticeable effects on the ileum were only found in one layer and the severity of damage varied greatly between individual birds, indicating that the reaction to DSS is variable in chickens. Although in the present study broilers had a much higher water intake than layers, the difference in water intake corresponded



**Figure 7.** Specific antibody titers against LPS and HuSA in response to an i.m. LPS/HuSA challenge in broilers and layers. DSS treated birds received 2.5 % DSS in drinking water from d 11 through d 18. Data are displayed as means and corresponding SE. Differences between treatment groups are indicated as follows: \*\* =  $P \leq 0.01$ , # =  $P \leq 0.1$ . Indicated differences indicate a main effect of treatment.

with the difference in BW so that water intake relative to BW was similar in both breeds and does probably not explain the differences in response to DSS between the two breeds. A great variability in the reaction to DSS is also found in rodents and may be influenced by a multitude of factors such as molecular weight of DSS, manufacturer, genetic susceptibility between species and strains, and environmental factors such as housing conditions (Perše and Cerar, 2012).

The present study also investigated the effects of DSS on ileal cytokine and immunoglobulin mRNA expression levels. The ileum is generally considered the site of immune activation and although DSS induces colitis, its effects are often not confined to the colon, but may also extend to other parts of the gastro-intestinal tract, including the ileum (Elsheikh et al., 2012). In rodents altered morphology and upregulation of pro-inflammatory cytokine expression levels throughout the small intestine were observed (Amit-Romach et al., 2006; Barada et al., 2006), supporting the notion that the effects of chemically induced colitis are not limited to the colon. Furthermore DSS induced colitis has also been described to lead to a systemic activation of the innate immune system (Westbrook et al., 2009). In contrast to the aforementioned rodent studies, effects of DSS on pro- and anti-inflammatory cytokine expression levels in the ileum were not observed in the present study with the exception of enhanced IL-1 $\beta$  expression levels in DSS treated layers after 7 days of DSS administration. Due to the findings in rodents it was expected that all ileal cytokine expression levels would be upregulated by DSS treatment. It should be noted, however, that cytokine expression levels in the present study were very close to or under the detection limit. It is also possible that effects of DSS in the ileum were localized more towards the terminal ileum and were not picked up in the present study, in which mid-section samples were investigated. For future experiments it would be interesting to investigate the effects on cytokine expression levels of the colon where more damage was observed.

DSS did have an effect on ileal immunoglobulin expression levels. Immunoglobulin expression levels were mainly affected in broilers with a down-regulation of IgM, IgY, and IgA expression levels in DSS treated broilers in the second half of the treatment period. The strongest effect was found for IgA, the most important immunoglobulin on mucosal surfaces, which was also the only immunoglobulin affected in layers. An earlier study indicated that the immune

system of broilers may be more humoral-oriented than that of layers (Simon et al., 2014), which may be the reason why the effects of DSS on immunoglobulin expression levels were more pronounced in broilers of the present study. Despite the lower Ig expression levels, no difference between DSS treated and control birds was found regarding expression levels of BAFF, a cytokine which seems to play an important role for B cell development and survival in species in which B cell development takes place in the GALT (Schneider et al., 2004; Kothlow et al., 2007; Yeramilli and Knight, 2010), indicating that at least on ileal level BAFF is not involved in the regulation of Ig expression levels in chickens. A speculative explanation for lower Ig expression levels in the ileum of DSS treated birds might be a possible migration of B cells towards the colon, the major site of DSS-induced pathology. Although differences between Ig levels between broilers and layers in the present study were not as pronounced as in the earlier study (Simon et al., 2014) and IgY expression levels were similar in both breeds, broilers still showed 4 times higher IgM and 3 times higher IgA expression levels on d 35 compared with layers. It should be kept in mind, however, that a higher or lower expression of mRNA does not necessarily mean a higher or lower synthesis of the respective protein.

DSS did have an effect on the antibody response towards the LPS/HuSA challenge which was administered two weeks after cessation of DSS treatment. Again effects were more pronounced in broilers where DSS treated birds showed lower antibody levels against LPS in response to the LPS/HuSA challenge. Interestingly broilers showed lower IgY antibody levels against LPS compared with layers which is in contrast with the earlier finding that broilers have a more humoral oriented immune system (Simon et al., 2014). On the other hand, broilers showed higher IgM antibody levels against HuSA on d 7 after administration of the challenge compared with layers. Similar differences between broilers and layers regarding their IgM and IgY antibody response towards an antigen were also observed by Koenen et al. (2002). In their study they found higher IgM antibody levels but lower IgY antibody levels against i.v. administered trinitrophenyl-conjugated KLH in broilers compared with layers. The fact that in the present study IgM antibody responses were only higher in broilers for HuSA but not LPS, and IgY antibody responses were only higher in layers for LPS but not HuSA might indicate that the differences that can be observed between the two breeds in terms

of antibody response depend on the administered antigen. In terms of DSS treatment no effects were found on antibody titers against HuSA. It is possible that the rather high dose of HuSA administered in the present study has led to a development of plateau levels of antibodies in all groups and by that eliminating treatment effects of DSS.

No effects of DSS on NAb levels (KLH) in response to the administered LPS/HuSA challenge were found. Since NAb levels were only determined in response to the administered LPS/HuSA challenge and not during DSS treatment, it is not clear whether DSS did not affect NAb levels or whether NAb levels have recovered in the two weeks between cessation of DSS treatment and administration of the LPS/HuSA challenge. Since NAb levels play a vital role in the first line of defense against pathogens and are considered an evolutionary preserved element of the immune system (Avrameas, 1991; Ochsenbein et al., 1999), it would be desirable to not be affected by transient intestinal pathologies or to recover quickly, making both options possible.

An interesting finding was that layers seemed to suffer more from DSS induced colitis in the present study compared with broilers and needed a longer time to recover regarding colon length and BW. One possibility why broilers seem to be less affected by DSS and show a faster recovery than layers may be a higher cell turnover and therefore faster tissue repair rate due to their rapid growth. Another possibility why broilers showed a faster recovery than layers may be differences between the two breeds regarding their immune system. As mentioned above, IL-1 $\beta$  expression levels in layers were upregulated in DSS treated birds in response to DSS after 7 days of DSS administration. In contrast IL-1 $\beta$  expression levels in broilers did not differ between DSS treated and control birds. This finding is in accordance with other studies indicating that layers seem to have a more pro-inflammatory way of responding as is indicated by higher pro-inflammatory cytokine expression levels attributed to innate immune cells in both spleen and ileum compared with broilers, while broilers seem to have a more humoral-oriented way of responding as indicated by higher ileal Ig levels compared with layers (Leshchinsky and Klasing, 2001; Simon et al., 2014). Studies in mice have shown that, together with IL-18, IL-1 $\beta$  plays an important role in the development of DSS-induced colitis and that treatment with an IL-1 receptor antagonist decreased the severity of symptoms (Siegmond et al., 2001). It is therefore possible that

layers show a more severe inflammatory response to DSS and bacterial antigens leading to more tissue damage, explaining the slower recovery and higher mortality rate in layers compared with broilers.

Studies in mice also suggest a genetic component in the development of DSS induced colitis, since different inbred mouse strains differ in their susceptibility to DSS (Mähler et al., 1998; Stevceva et al., 1999; Melgar et al., 2005).

Another possibility is that a different susceptibility to stress in combination with an immune system tending to more pro-inflammatory responses may be a reason for more severe symptoms, higher mortality and a longer recovery period in layers. That layers are more susceptible to stress compared with broilers has been suggested before as layers show higher plasma corticosterone levels and more stress-induced vocalizations in response to a stressor (Hocking et al., 1993; Saito et al., 2005). The longer recovery period of layers might also be in accordance with a higher susceptibility to stress, since a longer recovery period was also observed in DSS treated mice that were subjected to different stressors (social defeat and overcrowding) compared to DSS treated mice not subjected to those stressors (Reber et al., 2006). That stress may affect the severity of colitis induced by DSS and other chemicals, has previously been described in rodents where stress has been shown to reactivate quiescent colitis (Qiu et al., 1999; Melgar et al., 2008) and stress prior to or after induction of colitis has been shown to increase the severity of colitis and the accompanying symptoms (Gué et al., 1997; Reber et al., 2006). One of the causes for a stress-induced increase in severity of colitis may be the fact that stress affects the intestinal barrier function and thereby makes it possible for bacteria to adhere to and penetrate the intestinal epithelium (Qiu et al., 1999; Cameron and Perdue, 2005). Thus, stress seems to play an important role in the development of DSS induced colitis in chickens and should be taken into account in future studies.

Since a different susceptibility to stress has also been found for different chicken lines within one breed (Star et al., 2008), different chicken breeds and even different lines within one breed may require different doses of DSS to induce similar effects, which is in line with rodent studies (Mähler et al., 1998; Stevceva et al., 1999; Melgar et al., 2005). Regarding the appropriate dose of DSS in broilers, Menconi et al. (2015) recently suggested a tight dose range in chickens with a suggested optimum of 0.75 % DSS and therefore below the 2 – 5 % DSS used in

rodents (Perše and Cerar, 2012). The authors reported doses below 0.75 % DSS to be ineffective in causing disease symptoms, whereas higher doses caused such severe symptoms in broilers that experiments had to be terminated early. It is not known which broiler line was used in the study of Menconi et al. (2015) and discrepancies between their study and the present study in susceptibility to DSS may therefore be due to genetic differences in susceptibility in different broiler lines. Another point of consideration is that the environment may have an influence of DSS susceptibility as well (Perše and Cerar, 2012) and that the study of Menconi et al. (2015) was performed in a cage system while the present study was performed in a floor system.

Taken together, results of the present study show that DSS may be a useful model to induce intestinal pathology in chickens. Administered during immune development, DSS may furthermore have effects on antibody responses later in life. Results could further indicate that colitis induced by bacterial pathogens as is frequently observed in practice, may have long-term effects on the immune response. However, this aspect needs further investigation. Additionally it would be interesting to investigate the long-term effects of DSS under more challenging circumstances, i.e. higher antigenic pressure or in an infection model. More research is needed, however, regarding the appropriate dose, especially for layers that show a much more severe reaction to DSS compared with broilers. Additionally, possible effects of stress on DSS susceptibility in chickens deserve further attention.

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## REFERENCES

- Amit-Romach, E., R. Reifen, and Z. Uni. 2006. Mucosal function in rat jejunum and ileum is altered by induction of colitis. *Int. J. Mol. Med.* 18:721-727.
- Avrameas, S. 1991. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol. Today* 12:154-159.
- Barada, K., F. H. Mourad, S. I. Sawah, C. Khoury, B. Safieh-Garabedian, C. F. Nassar, and N. E. Saadé. 2006. Localized colonic inflammation increases cytokine levels in distant small intestinal segments in the rat. *Life Sci.* 79:2032-2042.
- Cameron, H. L., and M. H. Perdue. 2005. Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2. *J. Pharmacol. Exp. Ther.* 314:214-220.
- Cooper, H. S., S. N. S. Murthy, R. S. Shah, and D. J. Sedergran. 1993. Clinopathologic study of dextran sulfate sodium experimental murine colitis. *Lab. Invest.* 69:238-249.
- De Koning, D. B., E. P. C. W. Damen, M. G. B. Nieuwland, E. M. Van Grevenhof, W. Hazeleger, B. Kemp, and H. K. Parmentier. 2015. Association of natural (auto-) antibodies in young gilts with osteochondrosis at slaughter. *Livest. Sci.* 176:152-160.
- Dieleman, L. A., B. U. Ridwan, G. S. Tennyson, K. W. Beagley, R. P. Bucy, and C. O. Elson. 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 107:1643-1652.
- Dutra, R. C., R. F. Claudino, A. F. Bento, R. Marcon, É. C. Schmidt, Z. L. Bouzon, L. F. Pianowski, and J. B. Calixto. 2011. Preventive and therapeutic euphol treatment attenuates experimental colitis in mice. *PLoS ONE* 6:e27122.
- Elsheikh, W., K. L. Flannigan, W. McKnight, J. G. P. Ferraz, and J. L. Wallace. 2012. Dextran sulfate sodium induces pan-gastroenteritis in rodents: implications for studies of colitis. *J. Physiol. Pharmacol.* 65:463-469.
- Faure, M., C. Mettraux, D. Moennoz, J.-P. Godin, J. Vuichoud, F. Rochat, D. Breuillé, C. Obled, and I. Corthésy-Theulaz. 2006. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *J. Nutr.* 136:1558-1564.
- Gué, M., C. Bonbonne, J. Fioramonti, J. Moré, C. Del Rio-Lachèze, C. Coméra, and L. Buéno. 1997. Stress-induced enhancement of colitis in rats: CRF and arginine vasopressin are not involved. *Am. J. Physiol. Gastrointest. Liver Physiol.* 272:G84-G91.
- Hill, D. A., and D. Artis. 2010. Intestinal bacteria and the regulation of immune cell homeostasis. *Annu. Rev. Immunol.* 28:623-667.

- Hocking, P. M., M. H. Maxwell, and M. A. Mitchell. 1993. Welfare assessment of broiler breeder and layer females subjected to food restriction and limited access to water during rearing. *Brit. Poult. Sci.* 34:443-458.
- Hooper, L. V., D. R. Littman, and A. J. Macpherson. 2012. Interactions between the microbiota and the immune system. *Science* 336:1268-1273.
- Johansson, M. E. V., J. K. Gustafsson, K. E. Sjöberg, J. Peterson, L. Holm, H. Sjövall, and G. C. Hansson. 2010. Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model. *PLoS ONE* 5:e12238.
- Kerr, T. A., M. A. Ciorba, H. Matsumoto, V. R. T. Davis, J. Luo, S. Kennedy, Y. Xie, A. Shaker, B. K. Dieckgraefe, and N. O. Davidson. 2012. Dextran sodium sulfate inhibition of real-time PCR amplification: a poly-A purification solution. *Inflamm. Bowel Dis.* 18:344-348.
- Koenen, M. E., A. G. Boonstra-Blom, and S. H. M. Jeurissen. 2002. Immunological differences between layer- and broiler-type chickens. *Vet. Immunol. Immunopathol.* 89:47-56.
- Kothlow, S., I. Morgenroth, Y. Graef, K. Schneider, I. Riehl, P. Staeheli, P. Schneider, and B. Kaspers. 2007. Unique and conserved functions of B cell-activating factor of the TNF family (BAFF) in the chicken. *Int. Immunol.* 19:203-215.
- Kuttappan, V. A., L. R. Berghman, E. A. Vicuña, J. D. Latorre, A. Menconi, J. D. Wolchok, A. D. Wolfenden, O. B. Faulkner, G. I. Tellez, B. M. Hargis, and L. R. Bielke. 2015. Poultry enteric inflammation model with dextran sodium sulfate mediated chemical induction and feed restriction in broilers. *Poult. Sci.* 94:1220-1226.
- Laroui, H., S. A. Ingersoll, H. C. Liu, M. T. Baker, S. Ayyadurai, M. A. Charania, F. Laroui, Y. Yan, S. V. Sitaraman, and D. Merlin. 2012. Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. *PLoS ONE* 7:e32084.
- Leshchinsky, T. V., and K. C. Klasing. 2001. Divergence of the inflammatory response in two types of chickens. *Dev. Comp. Immunol.* 25:629-638.
- Lupp, C., M. L. Robertson, M. E. Wickham, I. Sekirov, O. L. Champion, E. C. Gaynor, and B. B. Finlay. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2:119-129.
- Mähler, M., I. J. Bristol, E. H. Leiter, A. E. Workman, E. H. Birkenmeier, C. O. Elson, and J. P. Sundberg. 1998. Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 274:G544-G551.

- Melgar, S., K. Engström, Å. Jägersvall, and V. Martinez. 2008. Psychological stress reactivated dextran sulfate sodium-induced chronic colitis in mice. *Stress* 11:348-362.
- Melgar, S., A. Karlsson, and E. Michaëlsson. 2005. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288:G1328-G1338.
- Menconi, A., X. Hernandez-Velasco, E. A. Vicuña, V. A. Kuttappan, O. B. Faulkner, G. Tellez, B. M. Hargis, and L. R. Bielke. 2015. Histopathological and morphometric changes induced by a dextran sodium sulfate (DSS) model in broilers. *Poult. Sci.* 94:906-911.
- Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* 286:2156-2159.
- Ohkusa, T., I. Okayasu, S. Tokoi, A. Araki, and Y. Ozaki. 1995. Changes in bacterial phagocytosis of macrophages in experimental ulcerative colitis. *Digestion* 56:159-164.
- Okayasu, I., S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, and R. Nakaya. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98:694-702.
- Perše, M., and A. Cerar. 2012. Dextran sodium sulphate colitis mouse model: traps and tricks. *J. Biomed. Biotechnol.* 2012:1-13.
- Qiu, B. S., B. A. Vallance, P. A. Blennerhassett, and S. M. Collins. 1999. The role of CD4<sup>+</sup> lymphocytes in the susceptibility of mice to stress-induced reactivation of experimental colitis. *Nat. Med.* 5:1178-1182.
- Reber, S. O., F. Obermeier, H. R. Straub, W. Falk, and I. D. Neumann. 2006. Chronic intermittent psychological stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* 147:4968-4976.
- Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9:313-323.
- Saito, S., T. Tachibana, Y.-H. Choi, D. M. Denbow, and M. Furuse. 2005. ICV CRF and isolation stress differentially enhance plasma corticosterone concentrations in layer- and meat-type neonatal chicks. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* 141:305-309.

- Schneider, K., S. Kothlow, P. Schneider, A. Tardivel, T. Göbel, B. Kaspers, and P. Staeheli. 2004. Chicken BAFF - a highly conserved cytokine that mediates B cell survival. *Int. Immunol.* 16:139-148.
- Siegmund, B., H. A. Lehr, G. Fantuzzi, and C. A. Dinarello. 2001. IL-1 $\beta$ -converting enzyme (caspase-1) in intestinal inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 98:13249-13254.
- Simon, K., G. De Vries Reilingh, B. Kemp, and A. Lammers. 2014. Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers. *Poult. Sci.* 93:1-11.
- Star, L., E. Decuyper, H. K. Parmentier, and B. Kemp. 2008. Effect of single or combined climatic and hygienic stress in four layer lines: 2. Endocrine and oxidative stress responses. *Poult. Sci.* 87:1031-1038.
- Stevceva, L., P. Pavli, G. Buffington, A. Wozniak, and W. F. Doe. 1999. Dextran sodium sulphate-induced colitis activity varies with mouse strain but develops in lipopolysaccharide-unresponsive mice. *J. Gastroenterol. Hepatol.* 14:54-60.
- Stevceva, L., P. Pavli, A. Husband, and W. Doe. 2001. The inflammatory infiltrate in the acute stage of the dextran sulphate sodium induced colitis: B cell response differs depending on the percentage of DSS used to induce it. *BMC Clin. Pathol.* 1:1-11
- Viennois, E., F. Chen, H. Laroui, M. T. Baker, and D. Merlin. 2013. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA *BMC Res Notes* 6:360-367.
- Westbrook, A. M., B. Wei, J. Braun, and R. H. Schiestl. 2009. More damaging than we think: systemic effects of intestinal inflammation. *Cell Cycle* 8:2482-2483.
- Yan, Y., V. Kolachala, G. Dalmasso, H. Nguyen, H. Laroui, S. V. Sitaraman, and D. Merlin. 2009. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. *PLoS ONE* 4:e6073.
- Yeramilli, V. A., and K. L. Knight. 2010. Requirement for BAFF and APRIL during B cell development in GALT. *J. Immunol.* 184:5527-5536.





# **CHAPTER 6**

## **General Discussion**



## INTRODUCTION

At hatch the chick's immune system is not fully mature yet, but will gradually develop during the first weeks of life (Jeurissen et al., 1989). When the protection by maternal antibodies has subsided at about two weeks post-hatch (Grindstaff et al., 2003; Hamal et al., 2006), the chick's own immune system needs to fully take over. The period early in life when the chick is relatively vulnerable due to the decrease of passive immunity, while the chick's own innate and adaptive immunity is not fully developed yet supposedly forms a critical window in immune development where shaping of future immune responses may be possible. This thesis therefore aimed to investigate to what extent early life factors such as feeding strategy, housing system, or disruption of intestinal homeostasis may influence the chick's immune development, but also its immune response later in life. Furthermore possible differences between two extreme chicken breeds, i.e. broilers and layers, were investigated. Earlier studies indicate that broilers and layers may show different responses towards immunological challenges, which may be due to unintentional co-selection of certain immunological traits along with selection for different production traits (meat and eggs, respectively) (Qureshi and Havenstein, 1994; Leshchinsky and Klasing, 2001; Koenen et al., 2002; Cheema et al., 2003).

This chapter will very briefly recapitulate performed experiments and subsequently discuss the findings presented in this thesis. Firstly, focus will be put on the importance of microbial colonization for immune development, and the consequences that delayed access to feed and administration of antibiotics can have on the microbiota composition will be discussed. Secondly, the effects of early feeding and dextran sulfate sodium on ileal immune development will be discussed, followed by the effects that different early life factors, e.g. access to feed post hatch or administration of antibiotics, can have on the specific antibody response later in life. Thirdly, observed differences between broilers and layers in terms of immune strategy and specific antibody response will be discussed, as well as differences in recovery from an intestinal pathology between the two breeds. Furthermore attention will be given to possible unintentional co-selection of immunological traits during selection for a certain production trait. Finally, limiting factors of the experiments conducted within the scope of this thesis will be

discussed, as will be the ongoing selection for production traits and the environmental limits to genetic selection.

## BRIEF RECAPITULATION OF EXPERIMENTS

All experimental treatments presented in this thesis, i.e. access to feed post hatch, housing conditions, administration of antibiotics, and induction of intestinal pathology by administration of dextran sulfate sodium (**DSS**), are expected to influence intestinal microbial colonization early in life. Due to the interaction between intestinal microbiota and the immune system, alterations in microbial composition during maturation and priming of the immune system early in life may affect immune responses later in life (Round and Mazmanian, 2009; Clarke et al., 2010). It has furthermore been proposed that the immune system does not only recognize specific antigens, but is also able to build up a nonspecific memory of danger based on cellular stress and damage experienced during immune maturation, which will subsequently determine the type of adaptive response in response to danger signals later in life (Noble, 2009).

In order to investigate the long-term influence of the aforementioned early life factors, chickens received a combination of *E. coli* derived lipopolysaccharide (**LPS**) and human serum albumin (**HuSA**) later in life, i.e. at 4 to 5 weeks of age or 15 weeks of age, depending on the experiment. The mainly innate response to the T cell independent LPS manifests itself in loss of bodyweight and sickness behavior. HuSA on the other hand is a T cell dependent antigen that elicits a specific humoral immune response. The adaptive response towards an LPS/HuSA challenge represents an important effector function. If this effector function is influenced by early life factors, this indicates that other parts of the immune system were presumably influenced as well. Results indeed show that the response to LPS/HuSA can be influenced by all investigated early life conditions. The implications and consequences of these results will be discussed below.

## INTESTINAL MICROBIOTA AND THE IMMUNE SYSTEM

In the studies presented in this thesis it was found that different early life factors may influence priming of the immune system during immune development, leading to altered antibody responses later in life. Based on our results and on findings in literature, all of these early life factors involve disturbances of early life intestinal microbial colonization. The intestinal microbiota is in close contact with the immune system and interaction between microbiota and the immune system shapes immune responses of the host.

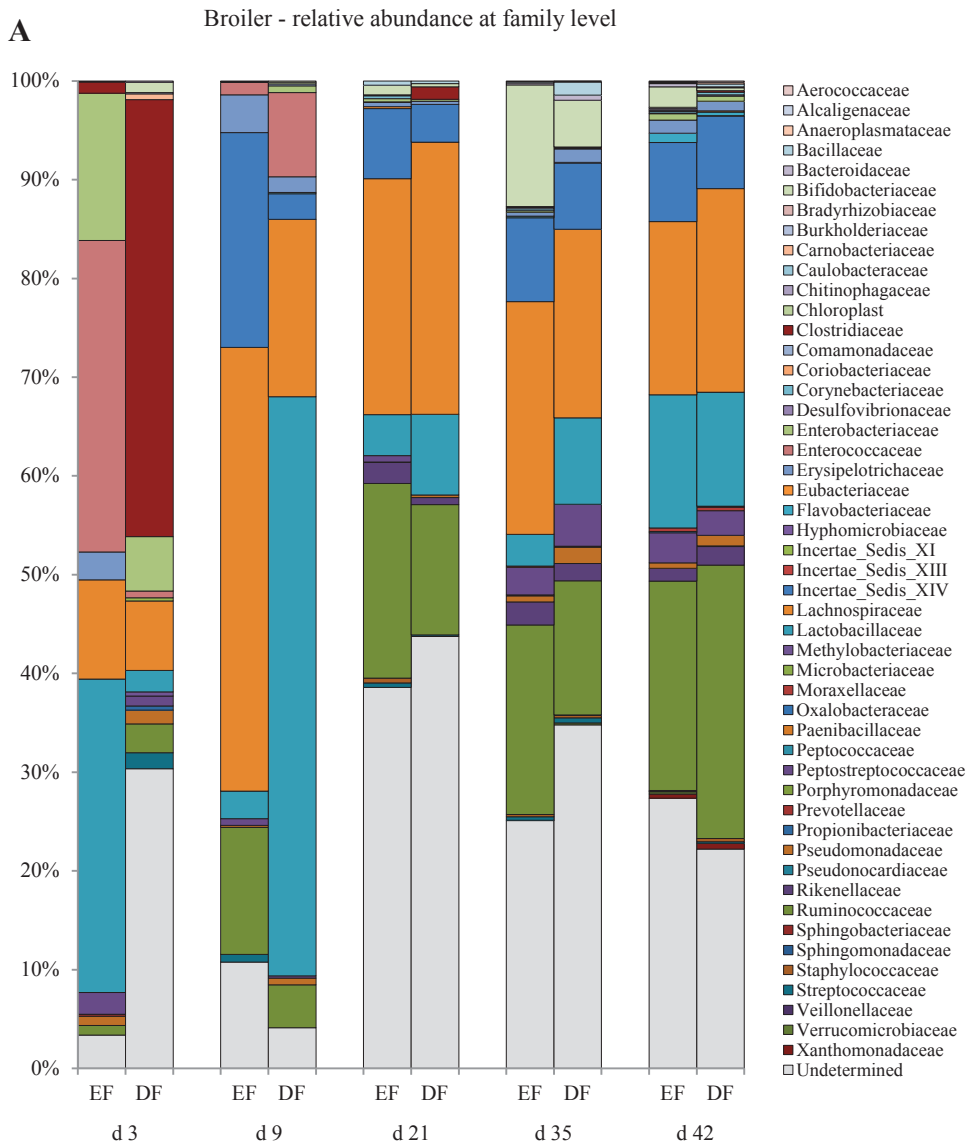
### *Importance of microbial colonization for immune development*

Studies in germ-free animals show the importance of adequate microbial colonization for gut and immune development. The intestine of germ-free animals is underdeveloped as these animals show a reduction in intestinal surface area, thinner intestinal cell walls, and a decreased cellular renewal in the small intestine to name just a few deviations from conventionalized animals (Smith et al., 2007). Similar to germ-free mice, also delayed fed chickens show a reduced intestinal surface area and lag behind in mucosal and villus development (Uni et al., 1998; Noy et al., 2001; Lamot et al., 2014), indicating the importance of adequate microbial colonization for gut development. Lack of intestinal bacteria not only leads to a delay in intestinal development, but also affects different parts of the intestinal immune system. Germ-free animals show for instance a reduction in production of secretory IgA, fewer and less cytotoxic intraepithelial lymphocytes, fewer and underdeveloped Peyer's patches, lower density of T cell subsets, and lower expressions of activation markers on macrophages (Barman et al., 1997; Smith et al., 2007; Round and Mazmanian, 2009). The effects of a lack of bacterial colonization are not confined to the intestine, but also have an effect on systemic immunity. Serum IgG levels are decreased in germ-free animals (Gustafsson and Laurell, 1958) as is the number of IgA and IgG secreting cells in various lymphoid tissues (Bos et al., 1988). Furthermore germ-free animals show lower specific antibody responses (Ohwaki et al., 1977) and susceptibility to various bacterial infections may be increased (Taylor et al., 1961; Inagaki et al., 1996). Immune development of germ-free mice can be stimulated by microbial components present

in the diet, such as lipopolysaccharide (Hrncir et al., 2008). Microbial colonization stimulates activation and terminal differentiation of B cells (Bos et al., 1987), and leads to an induction of class switching in B cells (He et al., 2007) and an increase of intestinal IgA plasma cells (Moreau et al., 1978). It has furthermore been shown in mice that the mucosal antibody repertoire develops in response to the intestinal microbiota (Hapfelmeier et al., 2010). In chickens it was recently found that gut microbiota are essential for bursal B cell development directly post hatch by forming immune complexes with maternal antibodies. These immune complexes are trapped in the bursa where they drive B cell differentiation and play a role in the induction of specific systemic IgM antibodies (Sonoda et al., 2013; Ekino et al., 2015). Additionally, microbial colonization is important for the induction of regulatory T cells and for maintaining a balance between Th1, Th2, Th17, and regulatory T cells (Mazmanian et al., 2005; Hall et al., 2008; Ivanov et al., 2008; Geuking et al., 2011), and the intestinal microbiota play a role in priming the innate immune system (Clarke et al., 2010).

### ***Effects of delayed feeding on ileal microbiota composition***

It has further been speculated that the initial microbiota composition may have profound effects on immune responses later in life, regardless of the mature microbiota composition (Maynard et al., 2012). Shapiro and Sarles (1949) have shown long ago that there is a dramatic increase in bacterial numbers in the intestine after the first intake of feed in the chicken. Delayed access to feed therefore very likely leads to differences in initial microbial colonization of the intestine which may have long-term consequences for immunity. To investigate the effects of early and delayed feeding on the adherent microbiota in the ileum, broiler and layer chicks either received immediate access to feed and water post-hatch or with a 72 h delay (chapter 2). At different time points ileal mid-sections of 1 cm were sampled between Meckel's diverticulum and caeco-iliac junction in 5 chickens per breed and feeding strategy. Ileal samples were carefully emptied of their contents, snap-frozen in liquid nitrogen and stored at -80° until further analysis. Adherent microbiota composition was determined by 16S rRNA pyrosequencing and mean relative abundance at family level is depicted in Figure 1. Undetermined families belong to the class of Clostridia.



**Figure 1.** Adherent microbiota composition in ileal mid-sections. Treatments were early feeding (EF, i.e. immediately post hatch) and delayed feeding (DF, i.e. with a 72 h delay). A: microbiota composition of broilers, B: microbiota composition of layers. (continued on the next page)

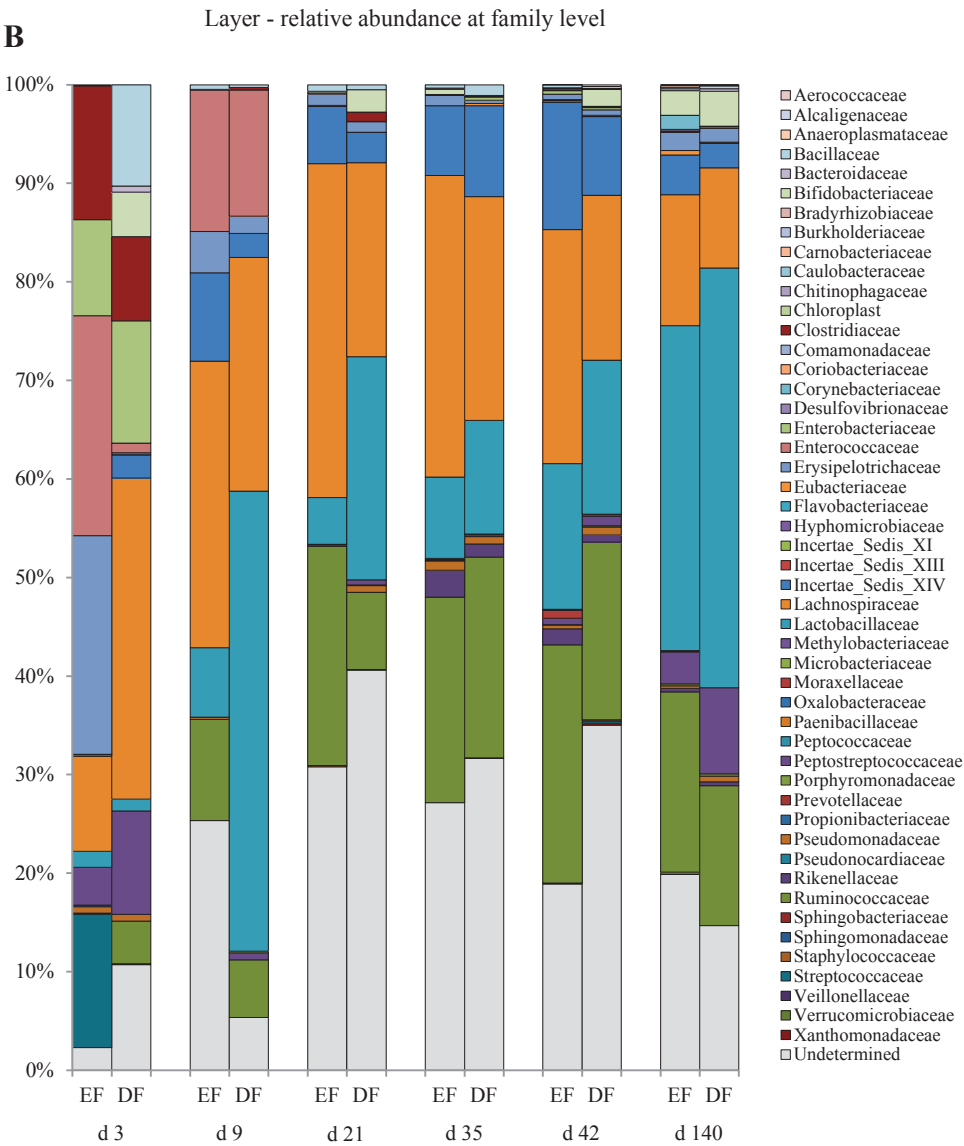


Figure 1. (continued)

Figure 1 shows that early and delayed fed birds of both breeds differ substantially in their microbiota composition on day 3 when delayed fed birds had not received access to feed yet. Data depicted in Figure 1 is intended to provide only a global overview on differences in microbiota composition between early and delayed fed chickens early in life and differences will not be discussed in detail. Differences in microbiota composition are still clearly visible on day 10, but microbiota composition becomes more similar between early and delayed fed birds from day 21 onwards in both breeds. Even though the microbiota composition becomes more similar, minor differences may still be biologically relevant in terms of stimulation of the immune system. It should also be noted that a large inter-individual variation in microbiota composition was observed. Although early life microbial colonization in chickens may also depend on antigens present in their environment, the data clearly shows that delayed access to feed affects ileal microbiota composition early in life, which may consequently lead to a different stimulation of the host immune system and differences in immunity later in life as has been shown in chapter 3. In that respect the offered diet itself may also influence microbial colonization. Differences and switches in diet lead to a shift in microbiota composition in humans and mice (Orrhage and Nord, 1999; Turnbaugh et al., 2009; De Filippo et al., 2010; Maslowski and Mackay, 2011) and diet-related differences in microbiota composition have also been described in chickens (Apajalahti et al., 2001; Knarreborg et al., 2002). Since initial microbial colonization seems to be important for immunity later in life, apart from provision of feed immediately post hatch, composition of the first feed offered to chicks after hatching may have profound effects on later life immunity as well.

### ***Disturbance of intestinal homeostasis***

Even if feed is provided immediately post hatch, disturbances of microbiota composition during the maturation phase of the immune system may have effects on later life immunity as was observed in chickens that received an antibiotic cocktail (chapter 4) or DSS (chapter 5) early in life. Both administration of antibiotics (Janczyk et al., 2007; Sekirov et al., 2008) and DSS (Okayasu et al., 1990; Faure et al., 2006; Lupp et al., 2007) lead to a shift of the intestinal microbiota composition. Especially the effects of administration of antibiotics on

microbiota composition and consequently the immune system have extensively been studied. Regarding antibiotics, already a single antibiotic dose has been shown to cause a long-lasting disruption of the intestinal microbiota composition (Janczyk et al., 2007; Schokker et al., 2015). The microbiota composition is often changed persistently and often does not entirely return to its pre-treatment composition, even after cessation of antibiotic treatment (Dethlefsen and Relman, 2011). The reason for long-lasting changes in microbiota composition is probably the fact that in an ecological community succession will take place whenever a niche becomes available due to a disturbance of some kind (Connell and Slatyer, 1977). Hereby early colonizers may have an influence on colonization by later species through modifying the environment (Connell and Slatyer, 1977). In case of the chicken disturbances may for instance include antibiotic treatment or intestinal pathologies.

In our study (chapter 4) microbiota composition of birds that were administered a multitude of antibiotics simultaneously for a longer period of time differed from control birds during administration of antibiotics. After the first week, the number of cultivable aerobic bacteria in feces were  $2.7 \times 10^3$  times lower in antibiotic treated birds compared with control birds. Furthermore fecal microbiota composition of antibiotic treated birds during and one day after cessation of antibiotic treatment differed significantly from control birds. In contrast to what has been reported by others, changes in microbiota composition were not long-lasting and microbiota composition of antibiotic treated birds was similar to that of control birds 2 weeks after cessation of antibiotic treatment. Nevertheless, initial microbial colonization of antibiotic treated birds differed substantially from control birds, leading to long-lasting effects on the specific systemic antibody response as discussed above. The importance of early life intestinal microbiota composition has been confirmed by others who showed that changes in microbiota composition in mice and humans affected numbers of invariant natural killer T cells in the intestine (Olszak et al., 2012) and led to development of allergic diseases later in life (Russell et al., 2012) only when disturbances were induced early in life, but not when disturbances were induced during adult life. Administration of antibiotics to chickens early in life may therefore disrupt the development regulatory immune functions and subsequently lead to a decrease in robustness.

The most evident difference between antibiotic treated and control birds in our study was an overpopulation with *Proteobacteria* and more precisely *E. coli* et rel. in antibiotic treated birds which accounted for more than half of their fecal microbiota, while the relative abundance of *Firmicutes* in these birds was diminished to less than half of that of control birds. These findings are in accordance with other studies which have shown that antibiotic treatment is often accompanied by a loss of colonization resistance, causing an increase in opportunistic potential pathogens belonging to *Proteobacteria*, while numbers of potentially beneficial bacteria belonging to *Firmicutes* often decrease (Van Der Waaij et al., 1971; Hengstes et al., 1985; Sullivan and Nord, 2001; Edlund and Nord, 2003; Fouhy et al., 2012). The observed differences in microbiota composition have likely led to differences in priming of the immune system, manifesting itself in the observed differences in the specific systemic antibody response. Certain lactobacilli, which were more abundant in control birds of our study, have been shown to enhance innate and adaptive immunity in mice (Gill et al., 2000; Kawase et al., 2012), direct T cell mediated immune responses (Mohamadzadeh et al., 2005), and attenuate inflammatory processes (Schultz et al., 2002; Osman et al., 2004; Pathmakanthan et al., 2004; Petrof et al., 2009). Furthermore certain lactobacilli are able to inhibit growth of potential pathogens such as *E. coli* or *S. aureus*, as has been shown for *Lactobacillus plantarum* (Gilliland and Speck, 1977; Gilliland, 1979). Clostridia, some of which were more abundant in control birds of our study, on the other hand have been found to be important for the maintenance of intestinal homeostasis by playing an important role in the induction of regulatory T cells (Atarashi et al., 2011; Lopetuso et al., 2013). Antibiotics used in our study can on the other hand lead to a reduction in colonic regulatory T cells (Atarashi et al., 2011) and a reduction of the innate immune defense, resulting in an increased susceptibility to enteric pathogens (Brandl et al., 2008).

### ***Implications and further research***

A balanced microbiota helps protect the host from invasion by enteric pathogens. Protective mechanisms include competition for nutrients and binding sites, release of antimicrobial molecules directed against a pathogen, and

stimulation of host defenses such as secretion of sIgA, defensins, or mucus (Stecher and Hardt, 2011). Disturbances of the initial microbial colonization of the chicken intestine may have long-lasting effects on specific systemic antibody responses and effects on other parts of the immune system cannot be excluded. Due to disinfection of eggs and chicks in commercial hatcheries, bacterial transfer between mother and offspring is largely prevented in current practice. For initial colonization post hatch chicks therefore depend on bacterial species present in their environment, and especially from feed for microbial colonization. Adequate microbial colonization in the first days of life is essential for the development of a functioning immune system due to the various interactions between the intestinal microbiota and the host immune system. Disturbances of microbial colonization should therefore be reduced to a minimum, especially during the phase of immune development. Since diet composition shapes the intestinal microbiota composition and the intestinal microbiota shape the development of the host immune system, dietary immunomodulation may be used to accelerate immune development. Especially in situations where infection pressure is high dietary stimulation of immune development might enhance chick survival. In the future it might be interesting to think about more customized solutions in terms of feed for housing conditions that differ in infection pressure.

## EARLY LIFE CONDITIONS INFLUENCE IMMUNE COMPETENCE

The gut is one of the most important sites from an immunological point of view. Within the gut the small intestine and especially the Peyer's patches (PPs) of the ileum play an important role for immune activation. Continuous sampling of the intestinal lumen content takes place via PPs, where encountered antigens are processed first (Hauet-Broere et al., 2003; Jung et al., 2010). PPs do not only play a role in the priming of immune cells and eliciting mucosal immune responses, but are also involved in the induction of oral tolerance and therefore play an important role in maintenance of homeostasis (Jung et al., 2010). The ileum is therefore often seen as the site of immune activation and education and effects of different early life factors on ileal immune development were investigated within the scope of this thesis. Although with a maximum of 5 PPs (Befus et al., 1980) chickens appear to

harbor less PPs compared to mammals, research indicates that chicken PPs also play a role in sampling antigen from the intestinal lumen (Hoshi et al., 1999).

### ***Limited effects of early feeding and DSS on ileal immune development***

Contrary to expectations, neither early feeding (chapter 2) nor DSS treatment (chapter 5) had an effect on ileal cytokine mRNA expression levels and had only very limited effects on ileal immunoglobulin mRNA expression levels. Feed intake is known to induce intestinal development and early feeding has been shown to increase intestinal cell proliferation and surface area (Uni et al., 1998; Noy et al., 2001; Lamot et al., 2014). Furthermore the chicken intestine is rapidly colonized by high numbers of bacteria immediately after the first ingestion of feed (Shapiro and Sarles, 1949). In the chicken a transient inflammation of the cecum and ileum characterized by an upregulation of pro- and anti-inflammatory cytokines in the first weeks of life may be necessary for immune activation (Lammers et al., 2010; Crhanova et al., 2011). It was therefore expected that early feeding would lead to an earlier onset in the upregulation of cytokines, which was not the case in our study (chapter 2). Similar to early feeding administration of DSS also did not lead to an earlier onset in the upregulation of ileal cytokines (chapter 5), although cytokine upregulation was expected in response to the inflammation caused by DSS. Also Ig expression levels were not greatly influenced by EF or DSS and an effect in terms of a down-regulation of Ig levels was mainly seen in broilers, which seem to be characterized by a more humoral oriented immune strategy (chapter 2).

Similar to our studies, Bar-Shira et al. (2005) found rather limited and transient effects of early feeding on the small intestine, while it led to a more rapid colonization of the hindgut and bursa with B and T lymphocytes. Furthermore it was shown in a study using fluorescent polystyrene beads that antigens taken up via the cloaca are mainly diverted to the bursa where B cell development takes place, but may also reach other parts of the intestine (Van der Sluis et al., 2009). Thus, due to its unique features that differ from those of mammals, in future studies the role of the chicken hindgut for immune education may be interesting to investigate as well.

***Early life conditions affect specific antibody responses later in life***

Despite the limited effects on ileal level observed in our studies, different early life conditions did influence the specific antibody response towards a combined LPS/HuSA challenge later in life. In a combination of different housing systems and feeding strategies, delayed fed birds raised in a floor system showed the most pronounced specific antibody response towards HuSA following i.t. administration of LPS/HuSA compared with cage housed birds, without an effect on the specific antibody response towards LPS (chapter 3). Administration of antibiotics early in life also affected the antibody response towards HuSA later in life in form of a suppression of the antibody response, again without an effect on the response towards LPS (chapter 4). In contrast, induction of intestinal pathology by administration of DSS did not affect the response towards HuSA, but did lead to lower antibody responses towards LPS (chapter 5).

Based on these experiments it can be concluded that early life conditions have long-term effects on the specific antibody response. The answer to why some experimental treatments affected the T cell dependent antibody response towards HuSA and others the T cell independent response towards LPS may lie within the different experimental designs. Delayed access to feed and administration of antibiotics affected microbial colonization in the immediate post-hatch period and led to a different start in life for animals in the experimental groups compared with control animals. Administration of DSS on the other hand led to intestinal pathology at a time when initial microbial colonization had already taken place, i.e. all animals had the same start in life. Another explanation may be the different immunization routes used in the experiments. Early and delayed fed birds in different housing systems as well as birds that received antibiotics early in life received LPS/HuSA intra-tracheally (mucosal immunization), while birds in the DSS study received LPS/HuSA intramuscularly (systemic immunization). Different immunization routes not only lead to different amounts of antibody production (Koenen et al., 2002; Bar-Shira et al., 2005; Murai et al., 2015), but may also cause the induction of different T cell responses (Xu-Amano et al., 1994). Oral administration of different T cell dependent antigens (mucosal immunization) was shown to preferentially induce Th2 responses, while intravenous or intraperitoneal administration (systemic immunization) induced Th1 responses in

different tissues. Different immunization routes in combination with different early life conditions may therefore have affected T cell dependent and independent antibody responses in a different way. An additional consideration why DSS treatment did not affect the specific antibody response towards HuSA is the relatively high dose of HuSA administered in the respective experiment (chapter 5), which may have led to the production of plateau levels of anti-HuSA antibodies in both treatment groups. The possible production of plateau levels of anti-HuSA antibodies may have camouflaged possible effects of DSS administration on the specific antibody response against HuSA.

Several mechanisms may have influenced the specific antibody response in the various experiments conducted. Early life feeding strategy, i.e. provision of feed immediately post hatch or with a 72 h delay, had more effect when birds were housed in a floor system than when they were housed in a cage system. As mentioned before, delayed fed birds in a floor system showed the most pronounced antibody response towards HuSA accompanied by the most pronounced sickness response following i.t. administration of a combination of LPS and HuSA (chapter 3). Others have looked at the effects of early feeding and housing system on the immune response as well, but so far the two factors had not been combined in one study. Apart from a more rapid colonization of the hindgut and bursa with T and B lymphocytes, early fed birds also mount higher primary antibody responses when immunized in the first week of life compared to delayed fed birds (Bar-Shira et al., 2005). These results seem to disagree with findings of our study where delayed fed birds showed a more pronounced primary antibody response, but the differences in experimental design presumably account for these seemingly inconsistent results. Birds in our study were immunized at 28 days of age, while birds in the study of Bar-Shira et al. (2005) were immunized at 6 days of age and delayed fed birds of that study may have been limited in their ability to react to the administered antigen, because at that time they still lagged behind in intestinal and bursal development. Additionally, maternal antibodies are still present in the first two weeks of a chick's life. Maternal antibodies may interfere with primary antibody responses in a suppressive way (Glezen, 2003). Although the presence of maternal antibodies may suppress primary antibody responses, priming of the immune system still takes place and the secondary response towards a booster immunization is not affected (Glezen, 2003). It would therefore have been

interesting to have investigated the effects of early and delayed feeding on the secondary response towards a booster immunization later in life when maternal antibodies had gone.

Regarding the influence of housing conditions on the immune response, it has been shown before that chickens kept in a floor system during the first weeks of life showed a higher specific antibody response towards a T cell dependent antigen later in life (Moe et al., 2010). Interestingly, in their study Moe et al. (2010) also showed that housing conditions after the rearing period, i.e. after 16 weeks of age, did not have an effect on the antibody response indicating the importance of early life housing conditions for later life immune responses.

Upon combining early life feeding strategies and housing systems it becomes clear that these two factors interact. Dibner et al. (1998) have postulated that delayed fed birds are impaired in their ability to cope with environmental and disease challenges as they show a drop in performance in response to an oral coccidiosis vaccine which was used as a model for a non-specific disease challenge. In our study delayed fed birds housed in a floor system showed a pronounced growth check in response to an intratracheal LPS/HuSA challenge (chapter 3). Based upon these findings, one might argue that in a floor system, in which antigenic pressure is high, delayed feeding may be disadvantageous. On the other hand these birds showed the highest response in terms of specific antibody titers against HuSA, indicating that these birds are very well able to cope with challenges, but that this comes at a cost in terms of performance. It is also likely, however, that the failure to develop tolerance in delayed fed birds led to a more pronounced antibody and sickness response. Oral tolerance towards (food) antigens is developed within the first week of a chick's life. It has been shown that tolerance can be induced in all chicks during the first 3 days of life, while tolerance induction is far less efficient after day 4 (Klipper et al., 2000). In order to develop long-term oral tolerance continuous exposure to the respective antigen in the first days of life is important (Klipper et al., 2000). In accordance with these findings, deprivation of dietary antigens and therefore lack of colonization with certain microbiota early in life may lead to an increased responsiveness towards LPS and a skew towards humoral immune responses (Da Silva Menezes et al., 2003; Sjörgen et al., 2009). Therefore chicks that were withheld from feed during the first 72 h post hatch may have failed to develop tolerance towards LPS, leading to an enhanced response

towards the LPS/HuSA challenge compared with birds fed immediately post hatch. The pronounced sickness response in delayed fed birds suggests that not only the humoral response has been affected, but delayed feeding has also had an influence on non-humoral parts of the immune system, since the sickness response is mediated mainly by pro-inflammatory cytokines (Johnson, 1998; Dantzer, 2001). If delayed access to feed in the immediate post hatch period has led to a failure to induce tolerance with a more pronounced response towards an antigenic challenge, then why did early access to feed not seem to matter in cage-housed birds? Cage-housed birds may have experienced more stress, since the cage environment prevents birds from performing natural behaviors such as dustbathing and foraging (Wang et al., 2003). Indeed, measurement of blood corticosterone levels in a very limited number of birds indicated higher corticosterone levels in cage-housed birds ( $1.2 \pm 0.4$  ng/ml) compared with floor-housed ( $0.8 \pm 0.1$  ng/ml) birds leading to the tentative speculation that the immune response might have been suppressed due to higher stress levels in cage-housed birds and was therefore similar between early and delayed fed birds in the cage system.

Delayed access to feed as well as administration of antibiotics during the first weeks of life lead to a reduction of antigenic stimulation and an altered microbiota composition in the intestine early in life. Opposite to delayed access to feed, administration of antibiotics did not lead to a more pronounced antibody response towards LPS/HuSA, but on the contrary dampened the antibody response. Despite the depletion of their microbiota, it is likely that birds that were administered antibiotics still developed tolerance towards LPS. LPS is used as an adjuvant and tolerance towards LPS may consequently lead to lower antibody responses towards a model antigen, in this case HuSA. It might seem contradictory to be able to develop tolerance towards LPS during a phase of microbiota depletion, but assessment of fecal microbiota composition in antibiotic treated birds revealed an overgrowth of *E. coli* in these birds during antibiotic treatment. As a consequence antibiotic treated birds were continuously exposed to a high amount of *E. coli* derived LPS early in life, which may have led to mucosal tolerance against *E. coli* derived LPS. Exposure to high amounts of *E. coli* derived LPS early in life has probably interfered with the *E. coli* derived LPS used as an adjuvant in the mucosally administered LPS/HuSA challenge later in life. Instead of acting as an adjuvant as is the case when LPS is administered simultaneously with or shortly

after a T cell dependent antigen (Luecke and Sibal, 1962; Merritt and Johnson, 1963; Hamaoka and Katz, 1973), LPS can act as a suppressor of T cell dependent antibody responses when it is administered some time before the model antigen (Luecke and Sibal, 1962; Merritt and Johnson, 1963; Persson, 1977; Parmentier et al., 2004; Maldonado et al., 2005). LPS has been suggested to directly enhance the suppressor function of regulatory T cells via the TLR-4 receptor (Caramalho et al., 2003), which has also been confirmed in chickens (Shanmugasundaram and Selvaraj, 2012). Additionally, dependent on the dose LPS may direct T cell responses towards a Th1 (high dose) or Th2 (low dose) response (Eisenbarth et al., 2002). A shift towards Th1 type responses may have taken place in antibiotic treated birds due to their exposure to high amounts of LPS during immune development. The exact mechanism underlying the observed effect in our chickens remains to be investigated, however, since studies in adult humans and rodents have shown that administration of antibiotics in a period in which immunization takes place may also suppress T cell dependent and/or T cell independent antibody responses, dependent on the antibiotic used (Hauser and Remington, 1982; Woo et al., 1999). Thus, although early life antibiotic treatment may influence immune development, antibiotic treatment later in life may also affect immune reactivity, independent of early life experiences.

In the experiments that investigated the effects of early feeding strategy and administration of antibiotics, the normal microbial colonization process was hampered in the experimental groups. In contrast, all chickens in the DSS experiment had the same start in life. In that experiment early feeding allowed for normal microbial colonization post hatch in all chickens, but intestinal pathology was induced in the second week of life. Nevertheless, the specific antibody response against LPS in response to the LPS/HuSA challenge later in life was affected by early life DSS treatment. Administration of DSS can lead to changes in microbiota composition (Okayasu et al., 1990; Faure et al., 2006; Lupp et al., 2007) and disruption of normal microbiota development early in life may influence immune development (Round and Mazmanian, 2009) and consequently lead to differences in immune responses later in life. Based on the findings in mice the microbiota of DSS treated birds may have been changed, but microbiota composition was not assessed in birds of the DSS experiment. Results indicate,

however, that intestinal pathology early in life may still influence immune responses later in life.

### ***Implications and further research***

Taken together, the results of the various experiments within the scope of this thesis and by others show that early life conditions may have an impact on the development of the (chicken) immune system and immune responses later in life. In this regard early life microbial colonization seems to be an important factor. During immune development, especially the first days seem to be of importance for tolerance induction, but the basis for later life immune responses may still be established to some extent in the days or weeks thereafter. Especially in high density situations on farms in which birds have to face high infection pressure good management procedures during the first weeks of life, such as early feeding or renouncing the use of antibiotics, may help with the development of a balanced immune system and increase robustness of animals.

Furthermore also immunological studies should take into account early life conditions as much as possible and not solely focus on the experimental treatment applied. In that respect it is also advisable not to purchase one day-old chicks for immunological studies, since those chicks are never exactly one day old, but may be between one and three days of age due to management procedures at commercial hatcheries. Purchase of one day-old chicks inevitable leads to some chicks being relatively early fed and chicks with a pronounced delay in access to feed.

## **A BROILER IS NOT A LAYER**

Chickens have been artificially selected for different production traits for decades and the selection is still ongoing. While layers have been selected for a high production of eggs, broilers have been selected for fast growth, i.e. meat production. Selection, be it artificial or natural, never affects just one single trait as has for instance been shown in domestication experiments (Trut et al., 2009). Selection for specific production traits has therefore very likely led to an

unintentional co-selection of other traits as well. That unintentional co-selection may include immunological traits has been confirmed in chapters 2 and 5.

### ***Immune strategy & specific antibody response***

It seems that broilers and layers have adapted different immune strategies during the selection process for different production traits. While broilers seem to have developed a more humoral-oriented immune strategy with high levels of immunoglobulins, layers seem to have developed a more cellular-oriented immune strategy with high levels of cytokines (chapter 2). Acute phase responses characterized by a massive release of cytokines are more costly (Husband, 1995; Iseri and Klasing, 2013) than antibody responses (Van Eerden et al., 2004; Korver, 2012) and since the fast growth of a broiler is energy-demanding it is possible that co-selection for a relatively cost-efficient immune strategy, i.e. investment in high amounts of antibodies rather than cytokines, has taken place. One should keep in mind, however, that results presented in chapter 2 were obtained on ileal level and may only reflect the situation on mucosal sites. Others have, however, also found lower pro-inflammatory cytokine levels in spleen cells of broilers compared with spleen cells of layers (Leshchinsky and Klasing, 2001). Furthermore in a splenocyte proliferation assay broilers, in contrast to layers, showed weaker non-specific cellular responses and a lack of specific cellular responses (Koenen et al., 2002), but the non-specific response of both breeds may depend on the mitogen used (Leshchinsky and Klasing, 2001). The breeds also differed in their specific systemic antibody responses to i.v. administered trinitrophenyl-conjugated KLH (Koenen et al., 2002). While broilers in that study showed higher IgM responses, layers showed higher IgY responses and maintained their antibody levels for a longer period of time. The breeds showed a larger difference in IgY antibody levels compared with IgM antibody levels and as an IgM response is part of the first line of defense against invading pathogens, the authors suggested that the IgM response is more important for host protection from an evolutionary point of view compared with the secondary IgY response and might therefore be less affected by selection.

***Recovery after intestinal damage***

Broilers and layers have also been shown to react differently to intestinal pathology in terms of intestinal damage and ability to recover (chapter 5). Intestinal pathology was induced by administration of DSS, which compromises the intestinal barrier. Several ways in which DSS affects the intestinal barrier function have been proposed. Firstly, DSS leads to an enhanced permeability of the intestinal mucus layer for bacteria, which consequently come in contact with the epithelial cell layer (Ohkusa et al., 1995; Stevceva et al., 2001; Johansson et al., 2010). Secondly, DSS decreases the capacity of macrophages to phagocytose bacteria, enabling bacteria to enter the lamina propria (Ohkusa et al., 1995; Stevceva et al., 2001). Furthermore, DSS may disrupt the intestinal barrier function through disruption of major cell pathways (Laroui et al., 2012). A direct toxic effect of DSS on epithelial cells has been suggested as well (Dieleman et al., 1994). All these effects of DSS lead to inflammatory responses, which include mucosal neutrophil infiltration and an upregulation of pro-inflammatory cytokines (Yan et al., 2009; Dutra et al., 2011). After administration of 2.5 % DSS in drinking water between day 11 and day 18 post hatch, layers showed a higher mortality and took a longer time to recover in terms of bodyweight and colon length, while broilers recovered quickly (chapter 5). Furthermore layers showed severe damage of different parts of the intestine. Layers showed damage and loss of the epithelial cell layer as well as loss of villi and crypts in colon and cecum, and in one case in the ileum as well. Broilers on the other hand showed no damage to the ileum, only a shortening and thickening of colonic villi and a partial loss of crypts in the cecum.

Differences in susceptibility to DSS have been reported for different inbred mouse strains, suggesting that genetic differences play a role in the development of DSS-induced colitis (Mähler et al., 1998; Stevceva et al., 1999; Melgar et al., 2005). Genetic differences between breeds might therefore explain the observed differences in susceptibility to DSS between broilers and layers. Layers that seem to have developed a more pro-inflammatory immune strategy compared with broilers (chapter 2) tended to show increased levels of IL-1 $\beta$  in response to DSS while broilers did not (chapter 5). In mice it was shown that IL-1 $\beta$  together with IL-18 plays an important role in the development of DSS-induced colitis and treatment with an IL-1 receptor antagonist alleviated symptoms (Siegmond et al.,

2001). Thus, the difference in immune strategy between the two breeds may have led to the fact that layers were more susceptible to DSS.

A different susceptibility to DSS between the two breeds does, however, not explain why both breeds hardly showed any reaction to DSS in a dose-response pilot study. A possible explanation for this discrepancy between pilot and follow-up study might be the supposedly more stressful environment of the follow-up study described in chapter 5, in which the birds had to deal with a slightly fluctuating climate, different caretakers and several changes in group composition, which was not the case in the pilot study. So rather than a difference in the susceptibility to DSS, one might speculate that a different susceptibility to stress might explain the more severe symptoms, higher mortality, and longer recovery period of layers compared with broilers in the follow-up study. The effect of stress on the severity of colitis induced by DSS and other chemicals has been shown in rodents, in which stress prior to or after induction of colitis increased the severity of colitis and the accompanying symptoms (Gué et al., 1997; Reber et al., 2006). Furthermore stress led to a reactivation of quiescent colitis in rodents (Qiu et al., 1999; Melgar et al., 2008). Comparative studies in broilers and layers indicate that layers are more susceptible to stress as they develop higher plasma corticosterone levels and show more stress-related vocalizations in response to stress induced by an open field test or restricted access to feed (Hocking et al., 1993; Saito et al., 2005). A possible explanation for the development of more severe colitis in stressed animals may be the fact that like DSS stress itself also affects the intestinal barrier function and makes it possible for bacteria to adhere to and penetrate the intestinal epithelium (Qiu et al., 1999; Cameron and Perdue, 2005). Therefore stress can enhance the detrimental effects of DSS and deserves consideration in future studies.

Except for differences in stress sensitivity, broilers may also suffer less from DSS and recover more quickly because of a higher cell turnover which comes with their fast growth. A higher cell turnover means that cells are renewed more quickly. Thereby it probably becomes harder for bacteria to penetrate further into the epithelial cell layer, which leads to less intestinal damage. The higher cell turnover of broilers makes it also possible to recover more quickly in terms of colon length and bodyweight after cessation of DSS treatment. On the other hand broilers in a very recent study showed severe symptoms of DSS induced colitis

when administered 1.5 % and 3 % DSS and the authors suggested that 0.75 % DSS would be a more appropriate dose to use in broilers (Menconi et al., 2015). Discrepancies between results obtained by Menconi et al. (2015) and the results obtained in our study may be due to higher stress levels in the birds of Menconi et al. (2015) which were kept in battery cages rather than in floor pens like birds in our study. Furthermore Menconi et al. (2015) do not state the broiler line they used in their study and genetic differences in susceptibility to DSS between different lines used in their study and our study may have played a role.

### ***Selection for a specific trait entails co-selection for other traits***

In the past it has repeatedly been demonstrated that selection for production traits comes at a cost of immunological traits and vice versa. In broilers and turkeys relative weight of primary and secondary immune organs decreased when birds were selected for increased bodyweight or increased egg production (Bayyari et al., 1997; Cheema et al., 2003). At the same time disease resistance was decreased (Yunis et al., 2000; Huff et al., 2005), although more so in the birds selected for high bodyweight than in birds selected for high egg production (Huff et al., 2005). Cutaneous basophil hypersensitivity responses to phytohemagglutinin-P (PHA-P) were also less pronounced in birds selected for high bodyweight, but were not influenced in birds selected for high egg production (Bayyari et al., 1997), indicating that immune responses are more affected by selection for high bodyweight than by selection for high egg production. Thus, the broiler immune system may have been compromised to a higher extent during the selection process for rapid growth than the layer immune system during the selection for high egg production.

Most studies do not compare broilers and layers, but investigate the consequences of selection for different traits within one breed. When broilers and layers are selected for higher bodyweights their antibody responses towards an antigen decrease (Miller et al., 1992; Qureshi and Havenstein, 1994; Cheema et al., 2003). Not only are the antibody responses lower in layers selected for high bodyweight, but antibody levels are also maintained for a shorter period compared with birds that have a lower bodyweight (Miller et al., 1992). Not all parts of the immune system seem to be affected in the same way by selection for bodyweight,

since in broilers selection for high bodyweight did not affect macrophage and NK cell functions in a study of Qureshi and Havenstein (1994). On the other hand Cheema et al. (2003) have reported that selection for high bodyweight was accompanied by higher phagocytic activity of macrophages, and higher cell-mediated and inflammatory responses and Yunis et al. (2000) postulated that the antibody response was independent of bodyweight. Discrepancies between findings may be caused by the fact that the selection process is still ongoing. Selection strategies and breeding goals may have changed over the years leading to different findings in older studies compared with more recent studies.

Not only does selection for production traits affect different parts of the immune system, but the other way around selection for certain immune parameters may affect production traits as well. In layers for instance, selection for high T cell dependent antibody responses against sheep red blood cells (SRBC) led to a decrease in bodyweight and egg production as well as an increase in the number of defective eggs (Siegel et al., 1982; Martin et al., 1990; Parmentier et al., 1998a; 1998b). While weights of primary and secondary immune organs were not affected, differences in resistance to various pathogens were observed (Gross et al., 1980). Higher antibody production against SRBC was accompanied with an increased resistance towards pathogens including a bacterium, a virus, and endo- as well as ectoparasites. Resistance was, however, not increased to all pathogens investigated, but was decreased to *E. coli* and *S. aureus*. In contrast to these findings, Siegel et al. (1982) have reported a lower mortality due to *E. coli* and *S. aureus* in birds with high antibody responses to SRBC and have suggested that contradictory findings may be due to differences in husbandry and the resulting gene x environment interactions. Additionally, different chicken lines within one breed may differ in their cellular and humoral immune responses even if they were selected for the same immunological trait (Van der Zijpp, 1983; Kreukniet et al., 1994) and it has been pointed out that a comparable selection protocol does not inevitably lead to a similar outcome in terms of immune responses (Kreukniet et al., 1994). In broilers on the other hand, selection for high antibody levels against *E. coli* did not necessarily compromise growth (Yunis et al., 2000). Higher mortality rates caused by infectious diseases were observed in birds with high growth rates, but were independent of antibody levels. Antibody levels did, however, become important in broiler lines with similar bodyweights, where low

antibody levels entailed higher mortality rates and the authors suggested a complex relation between growth rate, antibody levels towards *E. coli*, and resistance to infectious diseases. It would be interesting to investigate whether these interactions depend on the type of antibody response selected for, i.e. T cell dependent or T cell independent responses, or whether genetic differences between breeds play a role. It would furthermore be interesting to investigate other immune functions as well, since low antibody responses against *E. coli* may have been compensated by an enhanced innate response leading to a lower bodyweight in these birds despite their low antibody titers.

### ***Implications and further research***

In conclusion, a broiler is definitely not a layer as the two breeds show differences in terms of immunological traits, differ in their capability to handle intestinal pathology, and seem to differ in their susceptibility to stress. Selection for production traits, be it higher BW or higher egg production, may have profound effects on different parts of the immune system, depending on breed and line. This should be taken into account in breeding programs as well as in research projects aiming at immunomodulation via feed ingredients. Rather than aiming at an overall solution to create more robust chickens, one should consider whether tailor-made solutions might not be more desirable. As an example dietary compounds that stimulate immune development may be provided in husbandry situations with high antigenic pressure, whereas this might not be necessary under more hygienic situations.

## **FINAL REMARKS**

We have seen that broilers and layers differ largely, not only in their physical development, but also in their immune development and their reaction to immunological challenges. This means that there is no overall solution for generating a more robust chicken, but breeds and even lines within breeds differ in their needs, which calls out for more customized solutions, e.g. in terms of immunomodulation via feed. Furthermore the relationship between early life

conditions that affect microbial colonization and later life immune responses has been shown, illustrating the importance of adequate microbial colonization and a balanced microbiota composition. A balanced microbiota helps protect the host from invasion by pathogens through facilitation of rapid responses against potential pathogens via continuous modulation of the host immune system by the intestinal microbiota (Clarke et al., 2010; Stecher and Hardt, 2011; Willing et al., 2011).

### ***Limiting factors***

A limiting factor in the studies carried out within the scope of this thesis is that it is not possible to investigate all aspects of the immune system, which means that one always obtains only part of the picture. The immune system will always try to find some state of homeostasis, as this is the key to host survival. This means that a certain experimental treatment never just influences one part of the immune system, but other parts of the immune will inevitably be influenced as well. So while for example the adaptive arm of the immune system might be downregulated by a certain experimental treatment this does not automatically mean that an animal is less well protected as the shortcomings regarding the adaptive arm of the immune system might be compensated by an enhanced innate immune activity. This makes it difficult to judge whether for instance an enhanced specific antibody response is beneficial for an animal or not. On the other hand the interplay between various parts of the immune system is also an advantage when investigating long-term effects of early life conditions on host immunity, since alterations observed in one effector function such as the specific antibody response allow the conclusion that other parts of the immune system have very likely been altered as well.

Additionally all studies were performed in healthy animals and animals underwent a non-infectious immunological challenge. Under more practical situations with a higher stocking density and higher environmental antigenic pressure treatment effects may become more visible due to the fact that the animals may have less room in their adaptive capacity to deal with experimental challenges due to the several other challenges they already face under practical circumstances. Thus, under practical circumstances effects of early life conditions probably become even more pronounced.

### ***Ongoing selection***

A more general consideration is that selection for certain traits in different chicken breeds, but also in other farm animals is still ongoing. This means that results obtained now may not be true anymore in a couple of years. As discussed before, selection for certain production traits may also lead to selection for certain immunological traits. Additionally targeted selection for certain immunological traits takes place, since dampening of immunological responses leaves the animal with more resources to allocate to production traits such as growth. A problem we should be aware of is that with these selection procedures animals might be created that are tolerant to certain pathogens. Tolerance means that animals tolerate a pathogen to a certain extent without immunologically reacting to it, i.e. the animal does not waste resources on an immunological response but rather uses them for the production trait it was selected for. While in terms of economic profit tolerance seems like a good thing, it might not be such a good idea in terms of health and safety as animals might be selected to become tolerant to pathogens that can be harmful to humans. An aim should therefore be to obtain animals that are resistant to pathogens, i.e. animals in which infection is prevented or pathogenic load is decreased. Of course disease resistance may come at a cost of performance traits and the challenge is to find a good balance between robustness and performance of an animal. In that respect a fast and well-functioning innate arm of the immune system that is able to prevent clinical infections may be the key to both host health and performance. In that regard a more open dialogue between breeding companies and scientists would be desirable, since it is often not clear what the breeding goals of a company are and on which criteria selection is based. This is understandable from a breeding company's economic point of view, since competition is fierce. On the other hand a more open dialogue between breeding companies and scientists would save a lot of time and money on the scientists' side. By not knowing what animals are selected for, scientists are always one step behind in their research and may focus on ways to enhance an immunological response while selection criteria of breeding companies may aim at a lack of that specific immunological response. Thus, even more importantly, a more open dialogue could also save a substantial amount of experimental animals.

***Environmental limits to genetic selection***

Finally we should ask ourselves where we want to go in animal production and where the limitations lie. In a very interesting paper (Beilharz, 1998) stated that the limitation of animal production lies not within the genetic potential of animals. Instead resources provided by the environment are the limiting factor and genetic selection beyond these limitations will have detrimental effects. In other terms there is an environment-limited optimum to animal production and this optimum can only be increased when the environment is improved. The problem is that different environments entail a different optimum. Farm environments differ, for example in antigenic pressure due to differences in farm management, and what is optimal in terms of animal production traits for one farm environment may not be optimal or even be detrimental in another farm environment. This also becomes an issue when there is a mismatch between the environment in which the parent stock is selected and the environment the offspring has to face. An additional problem is that intensive genetic selection forces an animal to allocate resources to the production trait it was selected for, consequently diminishing the animal's adaptive capacity to react to unexpected environmental challenges (Rauw et al., 1998).

In conclusion we should ask ourselves whether we should still strive for more in terms of production traits or whether we should rather be satisfied with what has been achieved so far and face/accept the limits of animal production. As R.G. Beilharz (1998) has so nicely formulated: "Impossible goals are best recognized as impossible, before spending vast sums in documenting this empirically."

## REFERENCES

- Apajalahti, J. H. A., A. Kettunen, M. R. Bedford, and W. E. Holben. 2001. Percent G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. *Appl. Environ. Microbiol.* 67:5656-5667.
- Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, Y. Taniguchi, K. Takeda, S. Hori, I. I. Ivanov, Y. Umesaki, K. Itoh, and K. Honda. 2011. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* 331:337-341.
- Bar-Shira, E., D. Sklan, and A. Friedman. 2005. Impaired immune responses in broiler hatchling hindgut following delayed access to feed. *Vet. Immunol. Immunopathol.* 105:33-45.
- Barman, N. N., A. T. J. Bianchi, R. J. Zwart, R. Pabst, and H. J. Rothkötter. 1997. Jejunal and ileal Peyer's patches in pigs differ in their postnatal environment. *Anat. Embryol.* 195:41-50.
- Bayyari, G. R., W. E. Huff, N. C. Rath, J. M. Balog, L. A. Newberry, J. D. Villines, J. K. Skeeles, N. B. Anthony, and K. E. Nestor. 1997. Effect of the genetic selection of turkeys for increased body weight and egg production on immune and physiological responses. *Poult. Sci.* 76:289-296.
- Befus, A. D., N. Johnston, G. A. Leslie, and J. Bienenstock. 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J. Immunol.* 125:2626-2632.
- Beilharz, R. G. 1998. Environmental limit to genetic change. An alternative theorem of natural selection. *J. Anim. Breed. Genet.* 115:433-437.
- Bos, N. A., C. G. Meeuwssen, H. Hooijkaas, R. Benner, B. S. Wostmann, and J. R. Pleasants. 1987. Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. *Cell. Immunol.* 105:235-245.
- Bos, N. A., C. G. Meeuwssen, B. S. Wostmann, J. R. Pleasants, and R. Benner. 1988. The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. *Cell. Immunol.* 112:371-380.
- Brandl, K., G. Plitas, C. N. Mihu, C. Ubeda, T. Jia, M. Fleisher, B. Schnabl, R. P. DeMatteo, and E. G. Pamer. 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* 455:804-808.

- Cameron, H. L., and M. H. Perdue. 2005. Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2. *J. Pharmacol. Exp. Ther.* 314:214-220.
- Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express Toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 197:403-411.
- Cheema, M. A., M. A. Qureshi, and G. B. Havenstein. 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* 82:1519-1529.
- Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16:228-231.
- Connell, J. H., and R. O. Slatyer. 1977. Mechanisms of succession in natural communities and their role in community stability and organization. *Am. Nat.* 111:1119-1144.
- Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* enteritidis infection. *Infect. Immun.* 79:2755-2763.
- Da Silva Menezes, J., D. De Sousa Mucida, D. Carmona Cara, J. Isaura Alvarez-Leite, M. Russo, N. Monteiro Vaz, and A. M. Caetano de Faria. 2003. Stimulation by food proteins plays a critical role in the maturation of the immune system. *Int. Immunol.* 15:447-455.
- Dantzer, R. 2001. Cytokine-induced sickness behavior: where do we stand? *Brain Behav. Immun.* 15:7-24.
- De Filippo, C., D. Cavalieri, M. Di Paola, M. Ramazzotti, J. B. Poullet, S. Massart, S. Collini, G. Pieraccini, and P. Lionetti. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S.A.* 107:14691-14696.
- Dethlefsen, L., and D. A. Relman. 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci. U.S.A.* 108:4554-4561.
- Dibner, J. J., C. D. Knight, M. L. Kitchell, C. A. Atwell, A. C. Downs, and F. J. Ivey. 1998. Early feeding and development of the immune system in neonatal poultry. *J Appl Poult Res* 7:425-436.
- Dieleman, L. A., B. U. Ridwan, G. S. Tennyson, K. W. Beagley, R. P. Bucy, and C. O. Elson. 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 107:1643-1652.

- Dutra, R. C., R. F. Claudino, A. F. Bento, R. Marcon, É. C. Schmidt, Z. L. Bouzon, L. F. Pianowski, and J. B. Calixto. 2011. Preventive and therapeutic euphol treatment attenuates experimental colitis in mice. *PLoS ONE* 6:e27122.
- Edlund, C., and C.-E. Nord. 2003. Ecological impact of antimicrobial agents on human intestinal microflora. *Old Herborn University Seminar Monograph 7: Immune system and microflora*; eds. Heidt, P.J., Rusch, V., Van der Waaij, D.; Herborn Litterae:37-65.
- Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 196:1645-1651.
- Ekino, S., K. Sonoda, and S. Inui. 2015. Origin of IgM<sup>+</sup>IgG<sup>+</sup> lymphocytes in the bursa of Fabricius. *Cell Tissue Res.* 362:153-162.
- Faure, M., C. Mettraux, D. Moennoz, J.-P. Godin, J. Vuichoud, F. Rochat, D. Breuillé, C. Obled, and I. Corthésy-Theulaz. 2006. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *J. Nutr.* 136:1558-1564.
- Fouhy, F., C. M. Guinae, S. Hussey, R. Wall, C. A. Ryan, E. M. Dempsey, B. Murphy, R. P. Ross, G. F. Fitzgerald, C. Stanton, and P. D. Cotter. 2012. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob. Agents Chemother.* 56:5811-5820.
- Geuking, M. B., J. Cahenzli, M. A. E. Lawson, D. C. K. Ng, E. Slack, S. Hapfelmeier, K. D. McCoy, and A. J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 34:794-806.
- Gill, H. S., K. J. Rutherford, J. Prasad, and P. K. Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Br. J. Nutr.* 83:167-176.
- Gilliland, S. E. 1979. Beneficial interrelationships between certain microorganisms and humans: candidate microorganisms for use as dietary adjuncts. *J. Food Prot.* 42:164-167.
- Gilliland, S. E., and M. L. Speck. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. *J. Food Prot.* 40:820-823.
- Glezen, W. P. 2003. Effect of maternal antibodies on the infant immune response. *Vaccine* 21:3389-3392.

- Grindstaff, J. L., E. D. Brodie III, and E. D. Ketterson. 2003. Immune function accross generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proc. R. Soc. Lond., B, Biol. Sci.* 270:2309-2319.
- Gross, W. B., P. B. Siegel, R. W. Hall, C. H. Domermuth, and R. T. DuBoise. 1980. Production and persistance of antibodies in chickens to sheep erythrocytes. 2. Resistance to infectious diseases. *Poult. Sci.* 59:205-210.
- Gué, M., C. Bonbonne, J. Fioramonti, J. Moré, C. Del Rio-Lachèze, C. Coméra, and L. Buéno. 1997. Stress-induced enhancement of colitis in rats: CRF and arginine vasopressin are not involved. *Am. J. Physiol. Gastroint. Liver Physiol.* 272:G84-G91.
- Gustafsson, B. E., and C. B. Laurell. 1958. Gamma globulins in germ-free rats. *J. Exp. Med.* 108:251-258.
- Hall, J. A., N. Bouladoux, C. M. Sun, E. A. Wohlfert, R. B. Blank, Q. Zhu, M. E. Grigg, J. A. Berzofsky, and Y. Belkaid. 2008. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity* 29:637-649.
- Hamal, K. R., S. C. Burgess, I. Y. Pevzner, and G. F. Erf. 2006. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult. Sci.* 85:1364-1372.
- Hamaoka, T., and D. H. Katz. 1973. Cellular site of action of various adjuvants in antibody responses to hapten-carrier conjugates. *J. Immunol.* 111:1554-1563.
- Hapfelmeier, S., M. A. E. Lawson, E. Slack, J. K. Kirundi, M. Stoel, M. Heikenwalder, J. Cahenzli, Y. Velykoredko, M. L. Balmer, K. Endt, M. B. Geuking, R. Curtiss, K. D. McCoy, and A. J. Macpherson. 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* 328:1705-1709.
- Hauet-Broere, F., W. W. J. Unger, J. Garssen, M. A. Hoiijer, G. Kraal, and J. N. Samsom. 2003. Functional CD25<sup>-</sup> and CD25<sup>+</sup> mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *Eur. J. Immunol.* 33:2801-2810.
- Hauser, W. E., and J. S. Remington. 1982. Effect of antibiotics on the immune response. *Am. J. Med.* 72:711-716.
- He, B., W. Xu, P. A. Santini, A. D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D. M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. *Immunity* 26:812-826.

- Hengstes, D. J., A. J. Stein, S. W. Casey, and J. U. Que. 1985. Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: influence of antibiotics on colonization resistance. *Infect. Immun.* 47:118-122.
- Hocking, P. M., M. H. Maxwell, and M. A. Mitchell. 1993. Welfare assessment of broiler breeder and layer females subjected to food restriction and limited access to water during rearing. *Br. Poult. Sci.* 34:443-458.
- Hoshi, S., A. Uchino, K. Kusanagi, T. Ihara, and S. Ueda. 1999. Uptake of orally administered polystyrene latex and poly(D,L-lactic/glycolic acid) microspheres into intestinal lymphoid tissues in chickens. *Vet. Immunol. Immunopathol.* 70:33-42.
- Hrncir, T., R. Stepankova, H. Kozakova, T. Hudcovic, and H. Tlaskalová-Hogenová. 2008. Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. *BMC Immunol.* 9:65-75.
- Huff, G. R., W. E. Huff, J. M. Balog, N. C. Rath, N. B. Anthony, and K. E. Nestor. 2005. Stress response differences and disease susceptibility reflected by heterophil to lymphocyte ratio in turkeys selected for increased body weight. *Poult. Sci.* 84:709-717.
- Husband, A. J. 1995. The immune system and integrated homeostasis. *Immunol. Cell Biol.* 73:377-382.
- Inagaki, Y., T. Suzuki, K. Nomoto, and Y. Yoshikai. 1996. Increased susceptibility to primary infection with *Listeria monocytogenes* in germfree mice may be due to lack of accumulation of L-selectin+ CD44+ T cells in sites of inflammation. *Infect. Immun.* 64:3280-3287.
- Iseri, V. J., and K. C. Klasing. 2013. Dynamics of the systemic components of the chicken (*Gallus gallus domesticus*) immune system following activation by *Escherichia coli*; implications for the cost of immunity. *Dev. Comp. Immunol.* 40:248-257.
- Ivanov, I. I., R. de Llanos Frutos, N. Manel, K. Yoshinaga, D. B. Rifkin, R. Balfour Sartor, B. Brett Finlay, and D. R. Littman. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4:337-349.
- Janczyk, P., R. Pieper, W. B. Souffrant, D. Bimczok, H.-J. Rothkötter, and H. Smidt. 2007. Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. *ISME J* 1:180-183.
- Jeurissen, S. H. M., E. M. Janse, G. Koch, and G. F. De Boer. 1989. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* 258:119-124.

- Johansson, M. E. V., J. K. Gustafsson, K. E. Sjöberg, J. Peterson, L. Holm, H. Sjövall, and G. C. Hansson. 2010. Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model. *PLoS ONE* 5:e12238.
- Johnson, R. W. 1998. Immune and endocrine regulation of food intake in sick animals. *Domest. Anim. Endocrinol.* 15:309-319.
- Jung, C., J. P. Hugot, and F. Barreau. 2010. Peyer's patches: The immune sensors of the intestine. *Int J Inflam* 2010:1-12.
- Kawase, M., F. He, A. Kubota, K. Yoda, K. Miyazawa, and M. Hiramatsu. 2012. Heat-killed *Lactobacillus gasseri* TMC0356 protects mice against influenza virus infection by stimulating gut and respiratory immune responses. *FEMS Immunol. Med. Microbiol.* 64:280-288.
- Klipper, E., D. Sklan, and A. Friedman. 2000. Immune responses of chickens to dietary protein antigens I. Induction of systemic and intestinal immune responses following oral administration of soluble proteins in absence of adjuvant. *Vet. Immunol. Immunopathol.* 74:209-223.
- Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Appl. Environ. Microbiol.* 68:5918-5924.
- Koenen, M. E., A. G. Boonstra-Blom, and S. H. M. Jeurissen. 2002. Immunological differences between layer- and broiler-type chickens. *Vet. Immunol. Immunopathol.* 89:47-56.
- Korver, D. R. 2012. Implications of changing immune function through nutrition in poultry. *Anim. Feed Sci. Technol.* 173:54-64.
- Kreukniet, M. B., N. Gianotten, M. G. B. Nieuwland, and H. K. Parmentier. 1994. In vitro T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes. *Poult. Sci.* 73:336-340.
- Lammers, A., W. H. Wieland, L. Kruijt, A. Jansma, T. Straetemans, A. Schots, G. den Hartog, and H. K. Parmentier. 2010. Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. *Dev. Comp. Immunol.* 34:1254-1262.
- Lamot, D., I. B. Van De Linde, R. Molenaar, C. W. Van der Pol, P. J. A. Wijtten, B. Kemp, and H. Van Den Brand. 2014. Effects of moment of hatch and feed access on chicken development. *Poult. Sci.* 93:1-11.
- Laroui, H., S. A. Ingersoll, H. C. Liu, M. T. Baker, S. Ayyadurai, M. A. Charania, F. Laroui, Y. Yan, S. V. Sitaraman, and D. Merlin. 2012. Dextran sodium sulfate

- (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. *PLoS ONE* 7:e32084.
- Leshchinsky, T. V., and K. C. Klasing. 2001. Divergence of the inflammatory response in two types of chickens. *Dev. Comp. Immunol.* 25:629-638.
- Lopetuso, L. R., F. Scaldaferri, V. Petito, and A. Gasbarrini. 2013. Commensal clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog* 5:23.
- Luecke, D. H., and L. R. Sibal. 1962. Enhancement by endotoxin of the primary antibody response to bovine serum albumin in chickens. *J. Immunol.* 89:539-544.
- Lupp, C., M. L. Robertson, M. E. Wickham, I. Sekirov, O. L. Champion, E. C. Gaynor, and B. B. Finlay. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2:119-129.
- Mähler, M., I. J. Bristol, E. H. Leiter, A. E. Workman, E. H. Birkenmeier, C. O. Elson, and J. P. Sundberg. 1998. Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. *Am. J. Physiol. Gastroint. Liver Physiol.* 274:G544-G551.
- Maldonado, L. M. E., A. Lammers, M. G. B. Nieuwland, G. De Vries Reilingh, and H. K. Parmentier. 2005. Homotopes affect primary and secondary antibody responses in poultry. *Vaccine* 23:2731-2739.
- Martin, A., E. A. Dunnington, W. B. Gross, W. E. Briles, R. W. Briles, and P. B. Siegel. 1990. Production traits and alloantigen systems in lines of chickens selected for high or low antibody responses to sheep erythrocytes. *Poult. Sci.* 69:871-878.
- Maslowski, K. M., and C. R. Mackay. 2011. Diet, gut microbiota and immune responses. *Nat. Immunol.* 12:5-9.
- Maynard, C. L., C. O. Elson, R. D. Hatton, and C. T. Weaver. 2012. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489:231-241.
- Mazmanian, S. K., C. H. Liu, A. O. Tzianabos, and D. L. Kasper. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122:107-118.
- Melgar, S., K. Engström, Å. Jägervall, and V. Martinez. 2008. Psychological stress reactivated dextran sulfate sodium-induced chronic colitis in mice. *Stress* 11:348-362.
- Melgar, S., A. Karlsson, and E. Michaëlsson. 2005. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am. J. Physiol. Gastroint. Liver Physiol.* 288:G1328-G1338.
- Menconi, A., X. Hernandez-Velasco, E. A. Vicuña, V. A. Kuttappan, O. B. Faulkner, G. Tellez, B. M. Hargis, and L. R. Bielke. 2015. Histopathological and morphometric

- changes induced by a dextran sodium sulfate (DSS) model in broilers. *Poult. Sci.* 94:906-911.
- Merritt, K., and A. G. Johnson. 1963. Studies on the adjuvant action of bacterial endotoxins on antibody formation, V. The influence of endotoxin and 5-fluoro-2-deoxyuridine on the primary antibody response of the BALB mouse to a purified protein antigen. *J. Immunol.* 91:266-272.
- Miller, L. L., P. B. Siegel, and E. A. Dunnington. 1992. Inheritance of antibody response to sheep erythrocytes in lines of chickens divergently selected for fifty-six-day body weight and their crosses. *Poult. Sci.* 71:47-52.
- Moe, R. O., D. Guémené, M. Bakken, H. J. S. Larsen, S. Shini, S. Lervik, E. Skjerve, V. Michel, and R. Tauson. 2010. Effects of housing conditions during the rearing and laying period on adrenal reactivity, immune response and heterophil to lymphocyte (H/L) ratios in laying hens. *Animal* 4:1709-1715.
- Mohamadzadeh, M., S. Olson, W. V. Kalina, G. Ruthel, G. L. Demmin, K. L. Warfield, S. Bavari, and T. R. Klaenhammer. 2005. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc. Natl. Acad. Sci. U.S.A.* 102:2880-2885.
- Moreau, M. C., R. Ducluzeau, D. Guy-Grand, and M. C. Muller. 1978. Increase in the Population of Duodenal Immunoglobulin A Plasmocytes in Axenic Mice Associated with Different Living or Dead Bacterial Strains of Intestinal Origin. *Infect. Immun.* 21:532-539.
- Murai, A., K. Kitahara, S. Okumura, M. Kobayashi, and F. Horio. 2015. Oral antibiotics enhance antibody responses to keyhole limpet hemocyanin in orally but not muscularly immunized chickens. *Anim. Sci. J.* 87:1-9.
- Noble, A. 2009. Do we have memory of danger as well as antigen? *Trends Immunol.* 30:150-156.
- Noy, Y., A. Geyra, and D. Sklan. 2001. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poult. Sci.* 80:912-919.
- Ohkusa, T., I. Okayasu, S. Tokoi, A. Araki, and Y. Ozaki. 1995. Changes in bacterial phagocytosis of macrophages in experimental ulcerative colitis. *Digestion* 56:159-164.
- Ohwaki, M., N. Yasutake, H. Yasui, and R. Ogura. 1977. A comparative study on the humoral immune response in germ-free and conventional mice. *Immunology* 32:43-48.
- Okayasu, I., S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, and R. Nakaya. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98:694-702.

- Olszak, T., D. An, S. Zeissig, M. Pinilla Vera, J. Richter, A. Franke, J. N. Glickman, R. Siebert, R. M. Baron, D. L. Kasper, and R. S. Blumberg. 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336:489-493.
- Orrhage, K., and C. E. Nord. 1999. Factors controlling the bacterial colonization of the intestine in breastfed infants. *Acta Paediatr* 88:47-57.
- Osman, N., D. Adawi, S. Ahrne, B. Jeppsson, and G. Molin. 2004. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of *Lactobacillus* and *Bifidobacterium*. *Dig. Dis. Sci.* 49:320-327.
- Parmentier, H. K., W. J. A. Van den Kieboom, M. G. B. Nieuwland, G. De Vries Reilingh, B. N. Hangalapura, H. F. J. Savelkoul, and A. Lammers. 2004. Differential effects of lipopolysaccharide and lipoteichoic acid on the primary antibody response to keyhole limpet hemocyanin of chickens selected for high or low antibody responses to sheep red blood cells. *Poult. Sci.* 83:1133-1139.
- Parmentier, H. K., M. Walraven, and M. G. B. Nieuwland. 1998a. Antibody responses and body weights of chicken lines selected for high and low humoral responsiveness to sheep red blood cells. 1. Effect of *Escherichia coli* lipopolysaccharide. *Poult. Sci.* 77:248-255.
- Parmentier, H. K., M. Walraven, and M. G. B. Nieuwland. 1998b. Antibody responses and body weights of chicken lines selected for high and low humoral responsiveness to sheep red blood cells. 2. Effects of separate application of Freund's complete and incomplete adjuvant and antigen. *Poult. Sci.* 77:256-265.
- Pathmakanthan, S., C. K. F. Li, J. Cowie, and C. J. Hawkey. 2004. *Lactobacillus plantarum* 299: beneficial in vitro immunomodulation in cells extracted from inflamed human colon. *J. Gastroenterol. Hepatol.* 19:166-173.
- Persson, U. 1977. Lipopolysaccharide-induced suppression of the primary immune response to a thymus-dependent antigen. *J. Immunol.* 118:789-796.
- Petrof, E. O., E. C. Claud, J. Sun, T. Abramova, Y. Guo, T. S. Waypa, S.-M. He, Y. Nakagawa, and E. B. Chang. 2009. Bacteria-free solution derived from *Lactobacillus plantarum* inhibits multiple NF-KappaB pathways and inhibits proteasome function. *Inflamm. Bowel Dis.* 15:1537-1547.
- Qiu, B. S., B. A. Vallance, P. A. Blennerhassett, and S. M. Collins. 1999. The role of CD4<sup>+</sup> lymphocytes in the susceptibility of mice to stress-induced reactivation of experimental colitis. *Nat. Med.* 5:1178-1182.
- Qureshi, M. A., and G. B. Havenstein. 1994. A comparison of the immune performance of a 1991 commercial broiler with a 1957 randombred strain when fed "typical" 1957 and 1991 broiler diets. *Poult. Sci.* 73:1805-1812.

- Rauw, W. M., E. Kanis, E. N. Noordhuizen-Stassen, and F. J. Grommers. 1998. Undesirable side effects of selection for high production efficiency in farm animals: a review. *Livest. Prod. Sci.* 56:15-33.
- Reber, S. O., F. Obermeier, H. R. Straub, W. Falk, and I. D. Neumann. 2006. Chronic intermittent psychological stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* 147:4968-4976.
- Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9:313-323.
- Russell, S. L., M. J. Gold, M. Hartmann, B. P. Willing, L. Thorson, M. Wlodarska, N. Gill, M.-R. Blanchet, W. W. Mohn, K. M. McNagny, and B. B. Finlay. 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep.* 13:440-447.
- Saito, S., T. Tachibana, Y.-H. Choi, D. M. Denbow, and M. Furuse. 2005. ICV CRF and isolation stress differentially enhance plasma corticosterone concentrations in layer- and meat-type neonatal chicks. *Comp Biochem Physiol A Comp Physiol* 141:305-309.
- Schokker, D., J. Zhang, S. A. Vastenhouw, H. G. H. J. Heilig, H. Smidt, J. M. J. Rebel, and M. A. Smits. 2015. Long-lasting effects of early-life antibiotic treatment and routine animal handling on gut microbiota composition and immune system in pigs. *PLoS ONE* 10:e0116523.
- Schultz, M., C. Veltkamp, L. A. Dieleman, W. B. Grenther, P. B. Wyrick, L. Tonkonogy, and R. B. Sartor. 2002. *Lactobacillus plantarum* 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm. Bowel Dis.* 8:71-80.
- Sekirov, I., N. M. Tam, M. Jogova, M. L. Robertson, Y. Li, C. Lupp, and B. Brett Finlay. 2008. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect. Immun.* 76:4726-4736.
- Shanmugasundaram, R., and R. K. Selvaraj. 2012. In vivo-lipopolysaccharide injection alters CD4<sup>+</sup>CD25<sup>+</sup> cell properties in chickens. *J. Anim. Sci.* 90:2498-2504.
- Shapiro, S. K., and W. B. Sarles. 1949. Microorganisms in the intestinal tract of normal chickens. *J. Bacteriol.* 58:531-544.
- Siegel, P. B., M. N. Katanbaf, N. B. Anthony, D. E. Jones, A. Martin, W. B. Gross, and E. A. Dunnington. 1982. Responses of chickens to *Streptococcus faecalis*: Genotype-housing interactions. *Avian Dis.* 31:804-808.

- Siegmund, B., H. A. Lehr, G. Fantuzzi, and C. A. Dinarello. 2001. IL-1 $\beta$ -converting enzyme (caspase-1) in intestinal inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 98:13249-13254.
- Sjörge, Y. M., S. Tomicic, A. Lundberg, M. F. Böttcher, B. Björkstén, E. Sverremark-Ekström, and M. C. Jenmalm. 2009. Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses. *Clin. Exp. Allergy* 39:1842-1851.
- Smith, K., K. D. McCoy, and A. J. Macpherson. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* 19:59-69.
- Sonoda, K., K. Noguchi, and S. Ekino. 2013. Immune complexes of *E. coli* antigens and maternal IgG in the bursa of Fabricius. *Cell Tissue Res.* 354:813-821.
- Stecher, B., and W.-D. Hardt. 2011. Mechanisms controlling pathogen colonization of the gut. *Curr. Opin. Microbiol.* 14:82-91.
- Stevceva, L., P. Pavli, G. Buffington, A. Wozniak, and W. F. Doe. 1999. Dextran sodium sulphate-induced colitis activity varies with mouse strain but develops in lipopolysaccharide-unresponsive mice. *J. Gastroenterol. Hepatol.* 14:54-60.
- Stevceva, L., P. Pavli, A. J. Husband, and W. F. Doe. 2001. The inflammatory infiltrate in the acute stage of the dextran sulphate sodium induced colitis: B cell response differs depending on the percentage of DSS used to induce it. *BMC Clin Pathol* 1:1-11.
- Sullivan, Å., and C. E. Nord. 2001. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis* 1:101-114.
- Taylor, M. J., J. R. Rooney, and G. P. Blundell. 1961. Experimental anthrax in the rat: II. The relative lack of natural resistance in germ-free (lobund) hosts. *Am. J. Pathol.* 38:625-638.
- Trut, L., I. Oskina, and A. Kharlamova. 2009. Animal evolution during domestication: the domesticated fox as a model. *Bioessays* 31:349-360.
- Turnbaugh, P. J., V. K. Ridaura, J. J. Faith, F. E. Rey, R. Knight, and J. I. Gordon. 2009. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 1:1-10.
- Uni, Z., S. Ganot, and D. Sklan. 1998. Posthatch development of mucosal function in the broiler small intestine. *Poult. Sci.* 77:75-82.
- Van der Sluis, H. J., R. M. Dwars, J. C. M. Vernooij, and W. J. M. Landman. 2009. Cloacal reflexes and uptake of fluorescein-labeled polystyrene beads in broiler chickens. *Poult. Sci.* 88:1242-1249.

- Van Der Waaij, D., J. M. Berghuis-de Vries, and J. E. C. Lekkerkerk-van der Wees. 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J Hyg (Lond)* 69:405-411.
- Van der Zijpp, A. J. 1983. The effect of genetic origin, source of antigen, and dose of antigen on the immune response of cockerels. *Poult. Sci.* 62:205-211.
- Van Eerden, E., H. Van Den Brand, H. K. Parmentier, M. C. M. De Jong, and B. Kemp. 2004. Phenotypic selection for residual feed intake and its effect on humoral immune responses in growing layer hens. *Poult. Sci.* 83:1602-1609.
- Wang, W., R. F. J. Wideman, M. E. Chapman, T. K. Bersi, and G. F. Erf. 2003. Effect of intravenous endotoxin on blood cell profiles of broilers housed in cages and floor litter environments. *Poult. Sci.* 82:1886-1897.
- Willing, B. P., S. L. Russell, and B. Brett Finlay. 2011. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nature Reviews Microbiology* 9:233-243.
- Woo, P. C. Y., H.-W. Tsoi, L.-P. Wong, H. C. H. Leung, and K.-Y. Yuen. 1999. Antibiotics modulate vaccine-induced humoral immune response. *Clin. Diagn. Lab. Immunol.* 6:832-837.
- Xu-Amano, J., R. J. Jackson, K. Fujihashi, H. Kiyono, H. F. Staats, and J. R. McGhee. 1994. Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. *Vaccine* 12:903-911.
- Yan, Y., V. Kolachala, G. Dalmaso, H. Nguyen, H. Laroui, S. V. Sitaraman, and D. Merlin. 2009. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. *PLoS ONE* 4:e6073.
- Yunis, R., A. Ben-David, E. D. Heller, and A. Cahaner. 2000. Immunocompetence and viability under commercial conditions of broiler groups differing in growth rate and in antibody response to *Escherichia coli* vaccine. *Poult. Sci.* 79:810-816.





## Summary



A balanced immune system and the ability to mount an appropriate immune response where necessary are essential for survival and proper functioning of the body. The course for later life immune responses is set early in life during the developmental phase of the immune system and accordingly disturbances of immune development may have long-term consequences for host health. As the gut microbiota play an important role in immune activation and immune development, also disturbances of microbial colonization early in life may affect immune development and thus may have consequences for immune competence and disease resistance later in life. In chickens early life factors which are very likely to influence microbial colonization and therefore immune development include management procedures in commercial hatcheries, such as disinfection of eggs and chicks, and delayed access to feed post hatch, early life housing conditions, antibiotic treatment, and intestinal pathologies. The research performed within the scope of this thesis aimed to investigate in how far different early life conditions and interventions that are likely to disturb early life microbial colonization influence immune development as well as the specific humoral immune response later in life. Additionally, possible differences in immune development and immune response between broilers and layers were taken into account, since selection for different production traits may have entailed co-selection of immunological traits.

In chapter 2 the effect of immediate and delayed access to feed post hatch on immune development at ileal level was investigated. For this purpose 150 broiler hens (Ross 308) and 210 laying hens (Lohman Brown) either received immediate access to feed and water post hatch or with a 72 hour delay. Ileal cytokine and immunoglobulin mRNA expression levels were determined immediately post hatch and at 3, 6, 9, 14, 21, 35, and 42 days post hatch for both breeds and additionally at days 70 and 140 post hatch for layers. Bodyweight, relative spleen and bursa weights, as well as systemic natural antibodies against keyhole limpet hemocyanin (**KLH**) were assessed on the aforementioned days. Furthermore the number of ileal IgA-positive cells was determined in broilers after assessment in a limited number of samples in both breeds indicated a possible effect of early feeding in broilers.

As could be expected from literature, delayed fed birds of both breeds showed lower bodyweights than early fed birds. Yet, the effect of feeding strategy on all

other parameters was limited. Interestingly, however, broilers and layers differed in their ileal immune development in that broilers showed up to 28 times higher ileal immunoglobulin expression levels and up to 49 times lower ileal cytokine expression levels compared with layers.

Taken together the study presented in chapter 2 showed that early feeding did not seem to have a clear effect on ileal immune maturation, but also demonstrated that broilers and layers seem to differ considerably regarding ileal immune development, suggesting that the two breeds have adapted different immune strategies.

In chapter 3 the effect of immediate and delayed access to feed on the specific antibody response later in life was investigated in broilers that were kept in two different housing systems, since early feeding strategy might influence a bird's sensitivity towards its environment. In that study 128 broiler hens (Ross 308) received either immediate access to feed and water post hatch or with a 72 hour delay. Birds of both feeding strategies were either housed in a floor system containing wood shavings or in a cage system. On day 28 post hatch half of the birds of each feeding strategy and housing condition received one of two treatments: either a noninfectious lung challenge consisting of a combination of intratracheally administered *E. coli* derived lipopolysaccharide (**LPS**) and human serum albumin (**HuSA**), or intratracheally administered phosphate buffered saline (**PBS**) as a placebo. Specific antibody titers against LPS and HuSA, as well as natural antibody titers against KLH were determined on days -1, 0, 3, 7, and 14 relative to the challenge. Furthermore the birds' sickness response was assessed in the hours following administration of the LPS/HuSA challenge.

Delayed fed birds in the floor system showed the highest specific antibody response towards HuSA and the most pronounced sickness response in terms of growth check, sickness behavior and feeding motivation. Based on findings by others, it was hypothesized that delayed access to feed might have led to a skew towards humoral immune responses or an insufficient establishment of tolerance against LPS in these birds. In that regard immediate access to feed post hatch seemed to be of higher importance in a floor system as cage-housed birds of both feeding strategies did not differ greatly from each other. It is possible that the effects of immediate access to feed post hatch become more pronounced under

higher antigenic pressure from the environment. Another possibility is that in cage-housed birds immune responses were suppressed due to possibly higher stress levels in these birds.

Taken together, early life feeding strategy and housing conditions may have a long-lasting effect on immune responsiveness and should be taken into account in immunological studies.

In chapter 4 the effect of early life antibiotic treatment on fecal microbiota composition as well as the specific antibody response later in life was investigated. Antibiotic treatment was based on a protocol developed for use in rodents, which in mice leads to a phenotype resembling that of germ-free animals. To our best knowledge this protocol was adapted for use in chickens for the first time in our study. For this purpose 40 one-day-old laying hens (Lohman Brown) were orally administered a cocktail of broad-spectrum antibiotics during the first week of life followed by a milder antibiotic treatment during week 2 and 3. The number of aerobic cultivable bacteria in feces was assessed on day 8. Fecal microbiota composition was assessed on days 8, 22, 35, and 175. On day 105 all birds received a non-infectious lung challenge consisting of an intratracheally administered combination of LPS and HuSA. Specific antibody titers against LPS and HuSA were assessed prior to administration of the challenge on day 105 as well as 10 days after the challenge on day 115.

On day 8, i.e. at the end of administration of the broad-spectrum antibiotic cocktail, antibiotic treated birds showed  $2.7 \times 10^3$  times lower numbers of cultivable bacteria in their feces compared with control birds. Furthermore, antibiotic treated and control birds differed strongly in their fecal microbiota composition during antibiotic treatment, i.e. on days 8 and 22. Differences in fecal microbiota composition were most pronounced regarding the relative abundance of *Proteobacteria* and *Firmicutes*, with the vast majority of fecal bacteria belonging to *Proteobacteria* and especially *E. coli* et rel. in antibiotic treated birds, while in control birds the vast majority of fecal bacteria belonged to *Firmicutes*. Only two weeks after cessation of antibiotic treatment, however, no differences in fecal microbiota composition could be observed anymore between antibiotic treated and control birds. Yet, administration of antibiotics early in life did have an effect on the specific antibody response 12 weeks after cessation of antibiotic treatment and

antibiotic treated birds showed lower antibody titers against HuSA compared with control birds. It was hypothesized that the difference in microbiota composition may have affected immune regulatory functions and that the massive population with *E. coli* et rel. and the entailed high exposure to LPS early in life of antibiotic treated birds may have influenced the antibody response towards the LPS/HuSA challenge.

Taken together, the study showed that administration of antibiotics early in life could still affect the specific antibody response months after cessation of antibiotic treatment. Furthermore a dysbiosis of the intestinal microbiota early in life may potentially alter immune development and consequently immune responses in the long run, despite an apparently quick recovery of the microbiota composition takes place.

In chapter 5 the effect of an early life intestinal pathology on ileal immune development and the specific antibody response later in life was investigated. Administration of dextran sulfate sodium (DSS), which is widely used in rodent models of inflammatory bowel disease, was adapted as a model for intestinal pathology in chickens. For this purpose 130 broiler hens (Ross 308) and 130 laying hens (Lohman Brown) received access to feed and water immediately post hatch. From day 11 to day 18 post hatch half of the birds of each breed received 2.5 % DSS in drinking water. Control birds received plain drinking water. Relative ileal cytokine and immunoglobulin mRNA expression levels were determined on days 10, 11 (8 hours after the start of administration of DSS), 14, 18, and 35 post hatch. Further measurements included bodyweight on a weekly basis, cecum and colon lengths on days 18 and 35 post hatch, as well as histological observations of ileum, colon and cecum on day 18 post hatch. On day 35 post hatch, all birds received an intramuscular LPS/HuSA challenge and specific antibody titers were determined on the day before administration of the challenge and on d 3, 7, and 14 after administration of the challenge.

Ileal IL-1 $\beta$  expression levels were upregulated in DSS-treated layers on day 7 of DSS treatment, but not in broilers, indicating a more pro-inflammatory immune strategy of layers. Furthermore DSS treatment led to a downregulation of ileal immunoglobulin expression levels, although to a greater extent in broilers. Administration of DSS led to lower specific antibody titers against LPS in broilers,

while DSS treated layers tended to show higher IgM antibody titers against LPS. Interestingly, and in contrast to an earlier performed dose-response pilot study, layers showed more severe symptoms in response to DSS administration than broilers. Layers showed a higher mortality, took a longer time to recover in terms of bodyweight and colon length, and showed more intestinal damage in histological samples. It was hypothesized that the discrepancy between the pilot study and the follow-up study might have been due to a more stressful environment in the follow-up study and a higher susceptibility to stress in layers, since in rodents it was shown that stress could enhance the severity of symptoms in DSS-induced colitis.

Taken together, the study showed that when administered during immune development, DSS can affect specific immune responses later in life, and furthermore indicated that layers seem to have a more pro-inflammatory way of responding compared with broilers, leading to the development of more severe symptoms in these birds. Although more research is needed regarding the appropriate dose of DSS and possible effects of stress on DSS susceptibility in chickens, DSS-induced colitis may serve as a model to induce intestinal pathology in chickens.

Chapter 6 discussed, amongst others, the importance of undisturbed microbial colonization for immune development and the consequences that delayed access to feed post hatch and the administration of antibiotics early in life can have on the intestinal microbiota composition and consequently immune responses later in life. With respect to the effects of early and delayed access to feed post hatch on the intestinal microbiota composition new data was presented. Ileal microbiota composition was determined for 5 early fed (immediate access to feed post hatch) and 5 delayed fed (72 hour delay) broilers and layers, respectively, on days 3, 9, 21, 35, 42, and 140 (layer only) post hatch. Differences in ileal microbiota composition were clearly visible in both breeds on days 3 and 9, but microbiota composition became more similar between early and delayed fed birds of both breeds from day 21 onwards. The data clearly showed that early life feeding strategy affects ileal microbiota composition, which may consequently lead to a different stimulation of the host immune system and differences in immune responses later in life, as could be observed in the study presented in chapter 3.

Taken together, several conclusions can be drawn from the studies conducted within the scope of this thesis. First of all, it was found that early life factors such as access to feed post hatch and antibiotic treatment influence the intestinal microbiota composition and specific antibody responses later in life, which may be due to differences in immune development as a consequence of differences in microbial stimulation early in life. With respect to early feeding strategy, immediate access to feed post hatch seems to influence a bird's sensitivity towards its environment and in that regard may be of greater importance in an environment with higher antigenic pressure. Secondly, administration of DSS may be used as a model to induce early life intestinal pathology in chickens and when administered during immune development it may affect immune responses later in life, although more research is needed on the appropriate dose for chickens. Finally, broilers and layers seem to have adapted different immune strategies with layers responding in a more pro-inflammatory way than broilers. Thus, early life conditions as well as differences between broilers and layers should be taken into account in future immunological studies.





## About the Author



## **CURRICULUM VITAE**

Kristina Simon was born on the 7<sup>th</sup> of February 1983 in Gräfelfing, Germany. After graduating from high school (Dossenberger Gymnasium Günzburg) she worked as a veterinary assistant. After three years she decided to move to the Netherlands to study Animal Management at Van Hall Larenstein University of Applied Sciences in Leeuwarden. After obtaining her bachelor's degree she went on to Wageningen University where she obtained a master's degree in Animal Sciences. After that she worked as a research assistant for different chair groups at Wageningen University before starting her PhD research in April 2012. The results of this research are presented in this thesis.

## **PUBLICATIONS**

### **Refereed Scientific Journals**

- K. Simon, G. de Vries Reilingh, B. Kemp, A. Lammers. 2014. Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers. *Poultry Science* 93:3017-3027
- K. Simon, G. de Vries Reilingh, J.E. Bolhuis, B. Kemp, A. Lammers. 2015. Early feeding and early life housing conditions influence the response towards a non-infectious lung challenge in broilers. *Poultry Science* 94:2041-2048

### **Conference Book of Abstracts**

- K. Simon, L. Cornelissen, G. de Vries Reilingh, H.K. Parmentier, A. Lammers. 2012. Influence of early life microbiota composition on the circulating (auto-) antibody repertoire. Book of abstracts of the XII Avian Immunology Research Group Meeting, Edinburgh, Scotland, 28-31 August, 2012, p.52
- K. Simon, G. de Vries Reilingh, B. Kemp, A. Lammers. 2014. Effects of early feeding and housing system on ileal immune development and systemic immune response in broilers and layers. Book of abstracts of the XII Avian Immunology Research Group Meeting, Guelph, Canada, July 16-19, 2014, p.99
- K. Simon, B. Kemp, A. Lammers. 2015. Effect of DSS on intestinal morphology, immune development and immune response in chickens. Book of abstracts of the 66<sup>th</sup> Annual Meeting of the European Federation of Animal Science, Warsaw, Poland, August 31 – September 4, 2015, p. 539

## WIAS Training and Supervision Plan<sup>1</sup>

Description	Year
<b>The Basic Package (3.0 ECTS)</b>	
Ethics and Philosophy in Life Sciences, Dieren, The Netherlands	2012
WIAS Introduction Course, Wageningen, The Netherlands	2012
<b>International Conferences (4.2 ECTS)</b>	
XII Avian Immunology Research Group Meeting (AIRG), Edinburgh, Scotland	2012
14 <sup>th</sup> Gut Day, Leuven, Belgium	2012
XIII Avian Immunology Research Group Meeting (AIRG), Guelph, Canada	2014
66 <sup>th</sup> Annual Meeting of the European Federation of Animal Science (EAAP), Warsaw, Poland	2015
<b>Seminars and Workshops (1.5 ECTS)</b>	
WIAS Science Day, Wageningen, The Netherlands	2013-2015
The Future of Pre- and Probiotics, Wageningen, The Netherlands	2015
Workshop on Exploration & Sequencing of Human Microbiota: The Perfect Signature, Paris, France	2015
<b>Presentations (6.0 ECTS)</b>	
AIRG, Oral Presentation, Edinburgh, Scotland	2012
Oral Research Presentation, Boxmeer, The Netherlands	2013
CAWA Oral Presentation, Wageningen, The Netherlands	2013
WIAS Science Day, Poster Presentation, Wageningen, The Netherlands	2014
AIRG, Poster Presentation, Guelph, Canada	2014
EAAP, Oral Presentation, Warsaw, Poland	2015
<b>Disciplinary and Interdisciplinary Courses (2.7 ECTS)</b>	
(Immuno) PCR Course, Gothenburg, Sweden	2014
Advanced Immunology Course, Utrecht, The Netherlands	2015
<b>Advanced Statistics Courses (4.2 ECTS)</b>	
Advanced Statistics: Design of Experiments, Wageningen, The Netherlands	2013
Statistics for the Life Sciences, Wageningen, The Netherlands	2014
Generalized Linear Models, Wageningen, The Netherlands	2015
Mixed Linear Models, Wageningen, The Netherlands	2015

**Professional Skills Support Courses (6.2 ECTS)**

PhD Competence Assessment, Wageningen, The Netherlands	2012
Teaching and Supervising Thesis Students, Wageningen, The Netherlands	2012
Effective Behaviour in your Professional Surroundings, Wageningen, The Netherlands	2013
Project and Time Management, Wageningen, The Netherlands	2013
Techniques for Writing and Presenting a Scientific Paper, Wageningen, The Netherlands	2013
Survival Guide to Peer Review, Wageningen, The Netherlands	2015
Career Perspectives, Wageningen, The Netherlands	2015

**Research Skills Training (7.5 ECTS)**

Preparing own PhD Research Proposal	2012
Summer Course Poultry, Barneveld/Zeevolde/Eersel/Nunspeet, The Netherlands	2014

**Didactic Skills Training (9.5 ECTS)**

Lecture ADP-1	2014
Supervision of 1 BSc and 4 MSc Thesis Students	2012-2015
Supervision project group Introduction to the Animal Sciences	2012
Reviewing RMC proposals	2014

**Management Skills Training (3.0 ECTS)**

Organisation Committee WIAS Science Day, Wageningen, The Netherlands	2013
Organisation Immunoforce Workshop, Wageningen, The Netherlands	2013

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<b>Education and Training Total</b>	<b>47.8 ECTS</b>
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<sup>1</sup> 1 ECTS credit equals a study load of approximately 28 hours

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