Chicken intestinal development in health and disease

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Chicken intestinal development in health and disease

Transcriptomic and modeling approaches

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Thesis

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Abstract

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Intestinal health is an important condition for sustainable animal production. Since it is known that there is significant variation in intestinal health and functionality, there is much to gain in this respect. However, to fully exploit the biological potential of the animal's gastro-intestinal tract, the mechanism and regulation of major intestinal processes need to be unraveled first. In addition, identification of key components and processes involved in intestinal adaptation mechanisms may help to identify internal and external factors that influence the health and functioning of the gut. Improved knowledge in this area may contribute in defining rational strategies to improve sustainable animal production.

Traditionally research used reductionist approaches and focused on specific components or isolated processes related to intestinal functioning. However, the recent developments in the areas of genomics and computational sciences provide tools and methods that allow studying the system of the gut as a whole. In this thesis we have set first steps in the use of such Systems Biology approaches towards the identification of the key components and processes involved in intestinal functioning and health. We investigated molecular processes associated with gut development in chickens under two extreme contrasting conditions. We used an infection with *Salmonella* immediately after hatch and control animals to create the two contrasting phenotypic conditions. We used microarray-based genome-wide mRNA profiling to identify patterns of gene expression and cellular processes associated with each conditions. Comparisons between the two conditions and the application of modeling approaches revealed genes, groups of genes, molecular pathways, gene networks, and high level regulators of system behavior. We also used a mathematical modeling approach to describe the dynamics of cellular components of the immune system and their corresponding interactions under the same two contrasting conditions.

We identified different temporal gene expression profiles associated with morphological, functional and immunological processes. Several of these processes differed between the two contrasting conditions, whereas others were not affected be the experimental treatments. By inferring gene association networks, we observed that an infection with *Salmonella* considerably changes the behavior of intestinal tissue as well as the regulation of the underlying molecular processes. For each contrasting condition, we identified a specific set of potential high-level

regulator genes (hubs). We hypothesize that these hubs are steering systems behavior. Bioinformatic analysis of the hubs suggested that the disturbance with *Salmonella* is associated with a shift from transcriptional regulation in the non-disturbed tissue to cell-cell communication in the disturbed tissue. Furthermore, the generated mathematical model describes the dynamics of the cellular components of the immune system as well as the dynamics of the invading pathogen well. The model was able to predict the cellular immune response of the host against an invading pathogen.

We developed basic knowledge of (molecular) processes that are associated with different physiological conditions of intestinal tissue and we acquired global views on adaptation mechanisms of the intestine , including the regulation thereof. This information can be used to formulate new hypotheses about behavioral aspects of the gut, for the discovery of new biological mechanisms, and ultimately for the development of tools and rational strategies to improve intestinal functionality and health, either via diet and/or the host genotype. Such developments are urgently required to diminish the incidence and impact of intestinal diseases in farm animal species and to reduce the use of antibiotics in animal husbandry.

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General Introduction

1.1 Background

Due to the world's increasing population and the changes in diet preferences and lifestyle, the demand for animal derived food products is expected to increase rapidly. Annual meat production is expected to increase to 376 billion kilograms by 2030 (Report of a Joint WHO/FAO Expert Consultation). The expected demand for poultry meat will grow from 80 billion kilograms in 2005 to 132 billion kilograms per year in 2022. However, feeding more people requires a more efficient primary food production systems, the use of more sustainably produced feeds, increased feed efficiencies, and a significant reduction of the ecological footprint of livestock production. Better knowledge about the processes in the gastro-intestinal tract might help in designing these more efficient animal systems, with compromising animal health and welfare.

A disadvantage of the animal production systems developed in the past decade is the dependency on the use of growth-promoting antibiotics (GPA). Animals were given antibiotics, even without any clinical signs of an infection. In 2006 the use of GPA in animals was banned by the European Union, due to the risk of generating multiple drug resistant bacteria. In the Netherlands this culminated into the 'Verbond van Den Bosch' and a report 'Al het Vlees Duurzaam' (September 2011) based on the work by the commission van Doorn. The covenant describes the goal that by 2020 all meat in the supermarkets will be sustainably produced without the preventive use of antibiotics for intestinal infections, like diarrhea which occurs often in young animals. Improved understanding of processes in the gut and identification of the factors influencing these processes can significantly contribute to future developments required for the sustainable production of animal based food products without the preventive use of antibiotics. Because there is significant variation in intestinal functionality and health between animals, there is much to gain at the level of intestinal health and functionality. Therefore, to fully exploit the intrinsic biological potential of the animal's gut, more knowledge is required about the functioning of the intestine as a system.

The gut is the primary site for the intake, processing, conversion, and absorption of nutrients and other constituents in feed. Within the gut, a multitude of processes occur, which are involved in digestion, fermentation, nutrient absorption, nutrient metabolism, intestinal integrity, immune recognition, immune regulation and development of immune tolerance. All these processes are influenced by highly interacting factors such as the diet, the genetic background of the animal, and the residing microflora. Improved knowledge of these interactions and the functioning of the gut as a system may provide tools for modulating and improving animal

intestinal health and functionality by a combination of selective breeding, customization of animal nutrition, and active (intestinal) health management.
To describe the comprehensive description of molecular processes that are associated with two contrasting conditions of intestinal tissue of young chicken we applied -omics technologies. Dynamic changes of these functional, morphological and immunological processes during physiological variations may point to genes, pathways and physiological mechanisms that have an effect on the functional status and adaptation mechanisms of the intestine. In the experiments described in this thesis we used an infection with *Salmonella enterica* serovar Enteritidis (*Salmonella*) immediately after hatch to induce significant physiologic changes. In the thesis we also applied modeling and systems biology approaches to describe major system components and their quantitative relationships, especially those associated with immunological development under the two contrasting conditions: a disturbed (by *Salmonella*) and a non-disturbed developing intestine. The aim of this research was to provide basic knowledge, allowing to investigate how diverse factors regulate phenotypic variations in intestinal functionality and health of newly hatched chicken. The specific objective is to contribute to the fundamental understanding of the factors and mechanisms involved in the functioning and health of the system of the chicken gut tissue that result from the interplay between the host genotype, the residing microbes and pathogens, and feed in the gut.

1.2 Intestinal Functionality and Health

In the gut of animals, feed is digested and converted into energy sources that can be absorbed and transported across the gut epithelium. The microflora that reside in the gut encompass hundreds of different microbial species and play a major role in digestion, fermentation and metabolic conversions. The epithelial cell layer of the gut is strategically placed at the frontline between the luminal content and the underlying cells of the mucosal immune system. This physical barrier selectively permits the entry of nutrients while keeping out potentially harmful antigens and pathogenic microorganisms. The mucosal layers of the gut are associated with the largest number of immune cells in the body. Intestinal epithelial cells are constantly monitoring the antigen content of the gut and communicate with the underlying immune cells. Feed and intestinal microbes have a strong impact on this crosstalk. These complex interactions direct the development of a balanced intestinal system that has the ability to avoid excessive inflammatory responses to antigens and

commensal bacteria while it retains the capacity to defend the body against infections with pathogenic microorganisms.

Gastro-intestinal tract and digestion

The gastro-intestinal tract of poultry begins at the beak/mouth and ends at the vent (Figure 1.1). The process of the digestion of feed consist of the following consecutive steps: 1) Feed is gathered and broken down by the beak/mouth; 2) feed travels via de esophagus (tube between mouth and crop); 3) in the crop the feed is stored and moistened; 4) in the proventriculus (glandular stomach) digestive enzymes are produced and mixed with feed; 5) in the gizzard (muscular stomach) the feed is mechanical broken down; 6) the small intestine is specialized in enzymatic digestion of carbohydrates, proteins, fats, and absorption of nutrients, 7) in the caecum absorption of water from the fecal material takes place; 8) in the large intestine next to absorption of water from the fecal material, waste is stored and bacterial fermentation takes place (e.g. of undigested polysaccharides) to produce sugars; 9) the cloaca is the common chamber for digestive and urinary waste; 10) the vent is the exit for waste in the cloaca. The different compartments of the gut are associated with so-called 'gut-associated lymphoid tissue (GALT)'. The GALT is part of the mucosal immune system, and consists in chicken of the bursa of Fabricius (bursa), Peyer's patches and cecal tonsils [1-3].

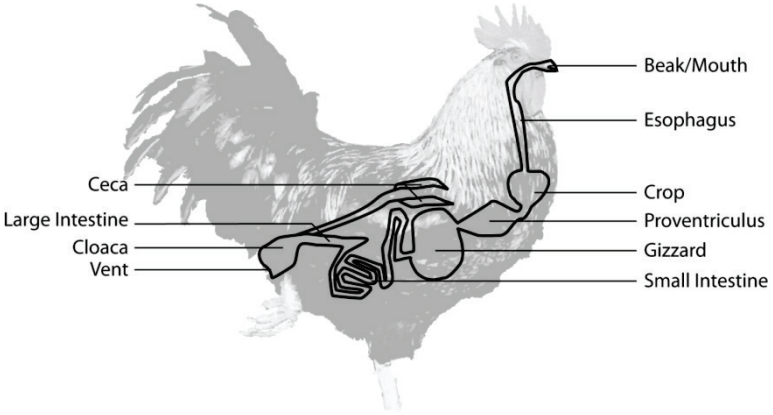

Figure 1.1 Schematic representation of the chicken gastro-intestinal tract
The main components of the gastro-intestinal tract are depicted. Important components for feed absorptions and digestion are shown from beak to vent.

Immune system

The immune system of vertebrates is involved in surveillance and protection against infectious invaders. Infectious invaders can be viruses, worms, fungi and bacteria. The immune system can be divided into two components, the innate and the adaptive immune system. The innate system provides the first line of defense and generates responses in a non-specific manner and acts immediately after challenge. The system is based on a limited, but diverse repertoire of receptors for antigen recognition. The adaptive part of the immune system acts as a second line of defense and is specific to invading organisms. In contrast to the innate system, mounting an adaptive response requires time. An important feature of adaptive immunity is immunological memory, which is established after initial exposure. When exposure re-occurs the adaptive systems responds faster and antibody affinity is increased.

Innate immune system

The innate immune system reacts directly against invading microorganisms by the activity of macrophages, Dendritic Cells (DC), Natural killer (NK) cells, γδ T cells, antimicrobial proteins and peptides, and the complement system. Because NK cells, antimicrobial peptides and the complement system are outside the scope of this thesis, they will not be described further here. The function of macrophages is to phagocytose cellular debris and pathogens. By expression of certain cytokines, macrophages stimulate other immune cells to respond to pathogens. DCs are specialized to process antigens and subsequently present these antigens on their surface to other immune cells. DCs have the ability to stimulate naïve T cells. They are also called professional antigen presenting cells and bridge the innate and adaptive immune system. In chickens DCs are present in the bursa, have a putative endocrine function and may be important for B cell induction [4].

The immune system of birds differs from mammals in structural and functional aspects, including the MHC architecture [5], birds lack lymph nodes [1] and difference in somatic generation of antibody diversity [6-9]. Birds have a different lymphocyte composition compared to mammals, especially with regard to the number of γδ T cells which is higher in spleen and peripheral blood of birds compared to mammals [10]. It has been observed that the number of γδ T cells in birds, but also in human, mice and cattle, rapidly increases after an infection with *Salmonella* [11-14]. Functions of γδ T cells include stimulation of immune defense mechanisms, acting as regulators by inhibiting the immune response, linking innate and adaptive immunity, as well as production of a wide variety of cytokines [14].

Adaptive immune system

T and B cells are the most prominent cells of the adaptive immune system. A variety of T cells exist, like Natural Killer T (NKT) cells, $CD4^+$ and $CD8^+$ T cells, T helper (Th) 1, Th2, and Th17, cytotoxic T cells and memory T cells. B cells are usually divided into plasma B cells and memory B cells. These different classes of T and B cells originate from a common ancestor, the lymphoblast, by the actions of a different combination of stimuli (cytokines) and interactions with antigens and virulence factors of pathogens. Here, a subset of cells will be described which are of importance for this thesis, including $CD4^+$ and $CD8^+$ cells.

Th1, Th2 and Th17 cells help to orchestrate an immune response against a variety of pathogens. Th1 cells are frequently associated with elimination of intracellular pathogens, whereas Th2 cells are involved in clearing parasitic worms, and Th17 cells with killing extracellular bacteria [15]. All these Th cells express the protein CD4 on their surface and are therefore also known as $CD4^+$ T cells. A minor subpopulation of $CD4^+$ cells which express CD25 on their surface, so called regulator T cells, are able to inhibit or suppress immune responses to self or invading antigens [16]. These cells control the immune response from going into overdrive. Another subset of T cells are cytotoxic T cells, also known as $CD8^+$ T cells. These $CD8^+$ are able to induce necrosis/apoptosis in cells infected with viruses or other pathogens and damaged cells. In chickens $CD8^+$ T cells play an important role in eliminating viruses, like infectious bronchitis virus (IBV) [17], reticuloendotheliosis virus (REV) [18], and Marek's disease virus (MDV) [19].

Cross-talk between innate and adaptive immune system

The innate and adaptive systems interact with each other via direct interactions between cells and via an array of signaling molecules, like cytokines. These cytokines, including interleukins and chemokines, are used for intercellular communication, are present in a large variety, and have different functions. Interleukins are the most important regulators of the immune system. Chemokines, attract immune cells to the site of infection (chemokine production). Certain cell types are capable of communicating with both the innate and adaptive system by direct cell-cell contacts, namely γδ T cells, NK T cells, DCs and B-1 cells. In most cases antigen presentation leads to activation of immune cells of the adaptive system, which produce either pro- or anti-inflammatory cytokines. Often cells have dual functionality, like NK cells which can lyse immature DCs and positively regulate DC maturation [20]. Thus the outcome of an interaction between the immune system and an invading pathogen is dependent on an array of environmental and intrinsic components and stimuli originating from the host as well as from the

invading pathogen. Therefore to understand the mechanisms involved in host-pathogen interactions at a systems level, it is important to monitor multiple biological levels simultaneously and perform analyses of the integrated data. Such approaches go beyond traditional reductionist approaches.

1.3 Intestinal Development in Chicken

Events occurring at early age have significant impact on the functioning of the intestine later in life [21, 22]. They may affect the morphological development, the functional development, and the immunological development of the intestine. Details of these developments are described in the next paragraphs. In Figure 1.2 a schematic overview is given of a cross section of the intestine, which shows villi, crypts and luminal content. Intestinal tissue consists of different cell types, including epithelial cell, goblet cells, Paneth-like cells, intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), T cells, B cells, DCs and macrophages [23]. Epithelial cells are covered with a mucus layer at the apical side, which protects the epithelial cells against aggressive luminal constituents including pathogenic micro-organisms [24]. The mucus layer consists of mucin proteins, which are produced and secreted by goblet cells. Stem cells residing in the crypts give rise to progenitor cells, which proliferate and differentiate while migrating to the tip of the villi. Under normal conditions epithelial cells are constantly sloughed off and replaced. In the lumen of the gut different (commensal) bacteria and feed particles are present. The luminal content is constantly monitored by the gut epithelial cells for the presence of harmful substances. When harmful substances are recognized and/or damage or cross the epithelial layer, immune signaling events lead to an immune response to eliminate the threat. Both feed and microorganisms interact with each other and with intestinal tissue and influence the morphological, functional and immunological development of the chicken intestine.

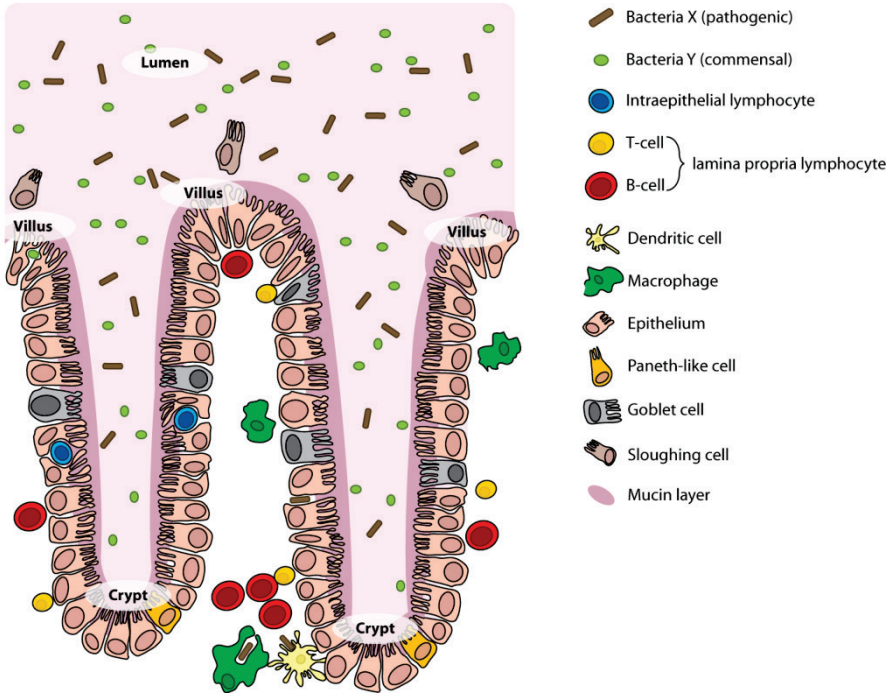

Figure 1.2 Simplified view of chicken intestinal structure

Different commensal and potential pathogenic microorganisms reside in the lumen. When these bacteria cross the epithelial layer, an immune response will be evoked to eliminate them. An array of immune cells is present in the intestine, including intraepithelial lymphocytes, T and B cells, dendritic cells and macrophages. The function of these cells is to monitor the luminal content and to eliminate invading bacteria and other unwanted microorganisms. The villi and crypts are composed of epithelial cells and goblet cells, and in crypts also an occasional Paneth-like cell is observed. Differentiation of enterocytes occurs from the crypt towards the villus. Also a mucus layer is present consisting of mucin proteins, which has a protective function.

Morphological development

In the small intestine of birds extreme morphological changes take place immediately after hatching. During that period the intestine gains more weight, relative to the whole body weight gain [25]. Small intestinal growth occurs in the absence and presence of feed, but the absolute and relative growth is lower when exogenous feed lacks [26]. Immediately after hatch the crypts begin to form and the number of crypts reaches a plateau after 2-3 days post hatch (dph) [25]. Villi increase rapidly in length during the first two days and in the jejunum a plateau is reached about 10 dph. Conversely the width of the jejunal villi increase marginally

and after 7 dph the maximum width is reached [27]. Also the density of jejunal villi increases during the first days and about 9 dph a constant level is reached [27]. Initially the epithelial enterocytes are non-polar and lack a defined brush-border membrane. They develop polarity, increase in length, and gain a distinguishable brush-border in the first 24 hours after hatch. Jejunal enterocytes further increase in length until 6 dph [27].

At villi tips cell death or apoptosis occurs, which corresponds to physiological turnover [28]. Apoptotic epithelial cells slough from the villi into the lumen, although macrophages may be involved in pushing these cells from the villi structures [29]. Distortions in the lumen of the gut lead to epithelial lesions and cause aberrant cell proliferation and extreme apoptosis. In chickens with malabsorption syndrome, heterophil infiltration and apoptosis play an important role in the pathogenesis. During malabsorption syndrome more apoptosis is observed in the villus tips compared to the villi tips of healthy chicken. Moreover infiltration of intestinal tissue/villi by heterophils points out acute inflammation, which is triggered by production of cytokines in the affected villus epithelium and LPLs [30].

Functional development

Migration of enterocytes from the crypt to villus tip is paired to differentiation of functions for digestion, absorption and mucin secretion [31]. For example the expression of all kinds of enzymes which are necessary for digestive functions. Major classes of intestinal digestive enzymes are disaccharidases and alkaline phosphatases [32, 33]. When the post-hatch transition from yolk-sac to exogenous feed occurs, disaccharidases are already present in the intestine and with aging the disaccharide activity increases [34]. Alkaline phosphatase is primarily expressed in villi tips and is considered to be a marker for enterocyte maturation [35, 36].

The capacity of birds to absorb carbohydrates is already detectable as early as embryonic day 18. At hatch birds have moderate absorption capacity and a few days after hatch the maximum absorption capacity of carbohydrates is reached [34]. This increasing absorptive capacity is due to the intestinal surface area expansion which occurs during morphological development. Thus the increase in absorptive surface results in a higher uptake of nutrients which is necessary for synthesis and growth of (other) tissues and organs. The digestive capacity in specific intestinal regions is associated to regional activity of mucosal enzymes.

Mucin protein, primarily acidic, is expressed from 17 days of incubation of the egg to 3 dph. After hatch both acidic and neutral mucins are produced by goblet cells [37]. The production of neutral mucin coincides with the colonization of the

intestine by microorganisms which interact with the mucus layer [38]. *In vitro* studies with intestinal cells of poultry and rats show that bacteria such as Lactobacillus strains adhere to intestinal mucin [39, 40]. In addition, competition for adhering to the epithelial/mucus layer occurs between commensal bacteria and pathogens [41, 42].

Development of Gut Associated Lymphoid Tissue (GALT)

After hatch, the GALT is colonized more rapidly with immune cells compared to other immune tissues. GALT maturation occurs probably in two stages, the first wave occurs during the first week post-hatch and a wave stage during the second week [43]. During the first wave increased expression of both IL2 and IFNγ is observed 4 dph, which is linked to the activation of T and NK cells. During the second wave, another increase of IL2 and IFNγ is observed, as well as an increase in the number of $CD3^+$ cells. This process of maturation is influenced by environmental stimuli, including feed and the developing microbial community. B cells colonize the intestine as early as 4 dph. [44]. During development the T cell composition of the GALT changes, where the IEL number of T cells expressing T cell receptor 1 (γ/δ) increases compared to TCR2 (α/β1) [45].

Colonization of precursor T cells in the avian thymus occurs in waves during embryogenesis [46], where three subpopulations are distinguished, namely γ/δ T cells, α/β1 T cells and α/β2 T cells [47]. In spleen, T cell colonization an the production of IL4, IL10, IL18 and IFNγ cytokines gradually increases during embryonic stages and post-hatch development with a maximum at 7 dph [48]. In avian species, B cell development occurs in three distinguishable phases: prebursal; bursal; and post-bursal [44]. For early B cell development CD79a expression is needed, whereas for later stages of B cell development CD79b expression is essential [49].

1.4 Host Response to *Salmonella*

Salmonella is a genus of rod-shaped, gram-negative bacteria in the family Enterobacteriaceae. The serovars of the species *S. enterica* and *S. typhimurium* cause the majority of food borne enteritis cases in humans. Although clinical symptoms are often lacking, one-day-old chicken infected with *Salmonella* do generate an immune response. There is a difference in the pathogenesis of *Salmonella* induced infections in one-day-old and mature chicken. In one-day-old chicken the immune system is immature and therefore these chicks are not able to clear he *Salmonella* infection. With only 10^7 CFUs a stable infection, measured in caecum, can be established [50]. In comparison, 14-day-old chicken have a

matured immune system and are able to clear an infection effectively. To establish a stable infection in 14-day-old chicken 10^9 CFU are needed [50].
The immune response of chicken to *Salmonella* has been studied in many different ways using traditional immunological approaches and using more advanced approaches such as investigating changes in gene expression of intestinal tissue. Although genome-wide approaches have been used, these studies primarily focus on genes associated to the host immune response to *Salmonella* [51-55]. These studies identified and characterized a number of different components involved in the (immunological) response of chicken upon a challenge with *Salmonella*. These components include immunological cells, signaling molecules, effector molecules, (signaling) pathways, and the products of a number of other genes. These studies showed that the expression of several immune-related genes, like IL2, IL10, IgL, avian beta-defensins (AvBDs) and TGFβ is significantly associated with the susceptibility of chicken to *Salmonella* [56-58]. For example, in susceptible young chickens the baseline level of AvBD gene expression is higher in intestinal (lymphoid) tissue and that of IFNγ is lower compared to chicken lines more resistant to a *Salmonella* carrier state [59]. In addition, chicken infected with *S. enteritidis* exhibit increased cytokine levels (CCLs, CXCLs and ILs) in caecum and spleen [60] and chicken infected with *S. typhimurium* show up-regulation of IL8, IL1β, K60 (CXC chemokine) and macrophage inflammatory protein 1β (MIP1β) in liver, spleen, jejunum, ileum, and cecal tonsils[61]. This suggests that both S. Enteritidis and *S. typhimurium* are capable of systemically infect chicken and that the host responds by initiating an immune response. Another approach to identify components involved in the susceptibility of chicken to *Salmonella* is to investigate whether single nucleotide polymorphisms (SNPs) differ in the genomes of susceptible and resistant lines. Such association studies also led to the identification of several genes contributing to *Salmonella* susceptibility [62, 63].
Salmonella has developed several different mechanisms to evade the host immune response. An important mechanisms is hiding within macrophages and crossing the epithelial barrier [64]. To this end *Salmonella* employs two different type 3 secretion system (T3SS), also known as 'molecular syringes', that inject T3SS effector proteins into host cell. The two T3SS systems are encoded by different gene clusters, *Salmonella* pathogenicity island (SPI)-1 and SPI-2 respectively. SPI-1 plays a role in the initial penetration of the intestinal mucosa, whereas SPI-2 is crucial for later systemic stages of infection [65].

1.5 Transcriptomics and Modeling

Microarray gene expression

The behavior (e.g. differentiation, morphogenesis, adaptation) of cells and tissues is defined by complex processes which involves many different components that operate at multiple biological levels. Ultimately this behavior is regulated by inter- and intra-cellular signaling molecules that modulate, directly or indirectly, the expression of genes. Expression of genes is regulated at the level of transcription by so-called transcription factors, and post-transcriptionally by small RNA molecules (miRNA, RNAi). The microarray technology appears very useful to generate snap-shot views of genome-wide gene expression profiles. However differentiation and morphogenesis of cells is controlled by the timing, location and dosage of gene expression. Therefore multiple snap-shot views in time are required as well as methods to analyze dynamic changes in cells and/or tissues based on these multiple transcription snap-shot views. Protocols for such approaches using complex tissues were not available at the start of the research described in this thesis. Different types of microarrays exist, the so called spotted and in situ synthesized arrays. For the first category, probes are synthesized prior to being spotted on the surface of the arrays, which can be silicone or glass. For the in situ synthesized arrays short oligonucleotide sequences, designed to represent a single gene or splice-variant, are synthesized directly on the array surface. Besides these different types of arrays, also different detection methods are used, e.g. by applying the two-color (double dye) or one-color (single dye) system. In the two-color method, Cy3 (green fluorescent) and Cy5 (red fluorescent) relative hybridization ratio's on a single array are used to detect whether genes are differentially expressed between two experimental conditions. In the one-color method, only Cy3 is used, and intensity levels are measured for the gene probes and compared between arrays. For the research described in this thesis the two-color arrays of ARK-genomics (Gallus gallus 20K v2 single spotted 20,460 oligonucleotides) were used, as well as the Agilent single color 4x44K chicken arrays (AMADID 15068), containing 43,451 probes. We used the sequentially transcriptomic data for: 1) generating spatiotemporal gene expression patterns, 2) inferring gene association networks, and 3) investigating early gene expression in chicken with different genetic background.

Bioinformatics, modeling and systems biology

Depending on the research questions to be answered, all kinds of approaches are used to analyze and interpret quantitative transcriptomic data providing snapshot views. In this thesis research I used the following tools; The Database for Annotation, Visualization and Integrated Discovery (DAVID) [66], Kyoto Encyclopedia of Genes and Genomes (KEGG) [67], GeneCards [68] and Search Tool for the Retrieval of Interacting Genes (STRING) [69]. Because DAVID is able to integrate information from many different databases, it is a useful tool for multiple analyses. For example DAVID can be used for identifying enriched Gene Ontology terms, visualization of genes on KEGG pathway maps, exploring gene names in batch, and converting gene identifiers. The combination of data derived from several databases is the major strength of such analyses I used this tool mainly to generate functional annotation of subsets or clusters of genes. Pathways from KEGG are used to extract (known and verified) gene-gene interactions. Subsequently our gene expression data was superimposed and visualized over time in Cytoscape. GeneCards is very useful for quick learning about the functional aspects of a gene, because of the integration of information about genes, proteins and diseases. STRING is used to investigate gene/protein interactions. The main advantage of this tool is that both experimental and predicted interaction information is available, even for more 'exotic' species. Studying interaction networks are used to get insight in gene regulatory mechanisms and to get a global impression of the biological consequences of changes in gene expression.

The availability of multiple snapshot views in time provides new opportunities to get insight into functional adaptation dynamics of tissues, e.g. functional analysis based on gene clusters displaying similar spatiotemporal expression patterns. The underlying hypothesis is that genes displaying similar spatiotemporal expression patterns belong to the same or closely related functional processes. Different tools/software are developed to investigate the interactions and/or regulation of genes, for example GeneNet [70, 71], WCGNA [72], or TimeDelay ARACNE [73]. Such gene association networks provide a glimpse of the complex interactions occurring on one biological level. A further step in inferring interaction networks is to generate interaction networks containing nodes of different biological levels, for example genes, proteins and metabolites. For bacteria and other organisms with relatively small genomes, metabolic networks have been constructed based on metabolite-metabolite interactions and their accompanying metabolic fluxes can be calculated. For the moment, heterogeneous tissues such as intestinal epithelium are too complex for such an approach, mainly because validation is difficult and laborious. In this thesis we set the first step in such an approach by describing

relationships between the differences in time-series transcriptomics data and differences in immunological, bacterial and weight data.
Besides the generation of interaction networks incorporating different biological levels, dynamics of a system can also be described by mathematical models. In the area of Systems Biology (SB) research focuses on the development of models representing the functionality of complex biological systems [74]. SB approach may provide more insight in the behavior of the biological system as a whole, because quantitative information of various biological levels are simultaneously examined and complex interactions between and within biological levels are taken into account. By generating quantitative data sets at different biological levels, e.g. transcriptomic data, influx of immune cells, bacterial counts, or metabolomics data, models can be constructed and subsequently validated. For generating such mathematical models different frameworks exist, like Boolean networks, Ordinary Differential Equations (ODEs) or cellular automata. These different frameworks all have their own characteristics for describing the system. In this thesis the focus was on ODEs, because we investigated cellular dynamics over time.

1.6 Objectives and Outline

The overall aim of this thesis is to identify and describe components and their interactions associated with intestinal development of young chicken under two contrasting environmental conditions: a non-disturbed and a disturbed condition as induced by an infection with *Salmonella*. This will provide basic knowledge allowing to investigate how diverse factors regulate phenotypic variation in intestinal health and functionality of newly hatched chicken. The specific objectives are: (I) Identify transcriptomic differences between healthy and *Salmonella* infected chicken intestines over time in order to identify major processes involved in chicken intestinal development as well as the influence of *Salmonella* on these developmental processes; (II) Investigate the differences in development and *Salmonella* response mechanisms of hatched chicken of three genetically different lines. (III) identify and characterize high level regulators of systems behavior under two contrasting conditions. Such high level regulators (hubs) are putative candidates for modulating intestinal behavior. (IV) Generate a first generation mathematical model representing major aspects of a healthy developing chicken intestine and a developing chicken intestine disturbed by an infection with *Salmonella*. Such a model may be applied to perform (*in silico*) simulations and predict the outcome of the system. Chapters 2, 3a, and 3b, are linked to objective I, chapter 4 is associated to objective II, and chapters 5 and 6 are associated with

objectives III and IV, respectively. In the following paragraphs a short outline of all chapters is presented.

The objective of Chapter 2 is to describe chicken jejunal development by investigating gene expression patterns in time. This approach has the potential to assign putative functions of biological processes underlying development. We hypothesize that gene expression patterns run parallel to immunological, morphological, and functional developments as measured by traditional methods. Therefore multiple time-points measurements were performed determining the gene expression and these gene expression patterns were clustered over time. For each cluster the general functionality of genes was generated by bioinformatic tools. Subsequently the different processes were categorized by their biological function.

In Chapter 3 and 4 we describe studies in which a similar approach was used as described in Chapter 2, however chickens were infected with *Salmonella* at day zero (hatch). The objective of Chapter 3 is to identify the effects of a severe disturbance on normal jejunal development in chicken at a global scale gene expression level. We investigated changes in chicken jejunal development at the gene expression level due to an infection with a pathogen and we focused on the time-ordered sequence of gene expression patterns and processes. We used *Salmonella* as a severe disturbing factor since in the chosen infection model it enters the systemic system by transmigration of the intestine and induces clear clinical effects and affects the intestine. For each cluster the general functionality of genes was generated by bioinformatic tools. Subsequently the different processes were categorized by their biological function. Furthermore the array data were verified by independent immunohistochemistry measurements. In chapter 3b the aim was to investigate which genes were correlated with the severity of systemic *Salmonella* infections over time. To this end a subset of the data described in Chapter 3 was used. For both the positive and negative correlating gene expression patterns subsequent bioinformatic analyses were performed.

The objective of the work presented in Chapter 5 was to investigate differences in the susceptibility of newly hatched chicks of 3 different commercial broiler lines to the systemic spread of *Salmonella* after oral infection and to identify the potential underlying mechanisms. Therefore three chicken lines with a genetically different background were investigated and different data were collected, including bacteriological examination, gene expression from intestinal tissue and immunohistochemistry of multiple time points. With these data statistical tests were performed to investigate significant differences in intestinal functionality and health dependent or independent of the genetic background of the host.

The objective of the work described in Chapter 6 is to make an effort to generate gene association networks describing the transcriptional response of chicken intestinal tissue in time under two highly contrasting conditions and to identify and characterize candidate high-level regulators. Temporal gene expression data were used to infer gene association networks (GANs) for both healthy and *Salmonella* infected chickens. Furthermore high-level 'regulators', so called hubs, were identified and subsequently characterized. By analyzing these GANs, the complexity of biological networks was investigated.

The objective of Chapter 7 was to construct a first generation mathematical model representing major aspects of the cellular immunological development and responsiveness during chicken intestinal development. In order to generate a mathematical model of a disturbed intestinal development we used developing chicken infected with *Salmonella*. The immune system represents a number of complex interactions at different biological levels and therefore deducing the immunological components will generate a model which can predict the cellular immunological dynamics of the intestine related processes over time. With this model simulations and perturbations regarding the chick intestinal immune system in time can be investigated *in silico*.

In Chapter 8 the results described in Chapters 2-7 are discussed in a broader context. I discuss the contribution of this research to the general understanding of the system in developing chicks in absence (control) and presence of a *Salmonella* infection. The results obtained are interpreted and the new approaches described herein are assessed. Lastly future development and recommendations are given.

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2

Gene Expression Patterns Associated with Chicken Jejunal Development

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Abstract

Jejunal development occurs in a spatiotemporal pattern and is characterized by morphological and functional changes. To investigate jejunal development at the transcriptomic level, we performed microarray studies in 1–21-day-old chickens. Nine gene clusters were identified, each with a specific gene expression pattern. Subsequently, groups of genes with similar functions could be identified. Genes involved in morphological and functional development were highly expressed immediately after hatch with declining expression patterns afterwards. Immunological development can be roughly divided based on expression patterns into three processes over time; first innate response and immigration of immune cells, secondly differentiation and specialization, and thirdly maturation and immune regulation. We conclude that specific gene expression patterns coincide with the immunological, morphological, and functional development as measured by other methods. Our data show that transcriptomic approaches provide more detailed information on the biological processes underlying jejunal development.

Key words: Microarray, Time-series, DAVID analysis, Immune system, Jejunum

2.1 Introduction

Development of the intestine occurs in the first week**s of life and can be categorized in morphological, functional and immunological development. Timing and onset of the underlying processes is not exactly known**. For instance, in the small intestine of birds extreme morphological changes occur directly after hatch. In the beginning crypts are undetectable and villi are undeveloped. After 2–3 days the number of crypts reaches a plateau [1]. Enterocytes that migrate from the crypt to the tip of the villus differentiate and mature to obtain functions for digestion and absorption [2], indicating that immediately after hatch morphological and functional developments begin.

Development of chicken intestinal function and structure seems to be correlated with development of the gut-associated lymphoid tissue (GALT), as observed in delayed feeding experiments [3]. The immune system consists of two main components, innate (non-specific) immunity and adaptive (specific) immunity. The innate response is characterized by direct reaction after exposure and absence of immunological memory. The adaptive immune response has a lag time between exposure and response and does generate immunological memory. At hatch immature T and B-cells, components of adaptive immunity, are already observed in the GALT and functional maturation occurs during the first 2 weeks [4]. In addition, independent of exposure to feed and microorganisms, local extramedullary hematopoiesis of granulocytes occurs in the small intestine as shown by histology and gene expression (PSEN1, b-defensin) [5]. The gene expression of proinflammatory cytokines that is observed immediately after hatch is dependent on the presence of microorganisms and feed. Recruitment of lymphocytes later in life is related to the proinflammatory activity in the developing intestine [5]. Thus, immunological development of the intestine occurs in the first 2 weeks, when the intestinal immune function is maturing due to feed exposure.

To investigate jejunal development in poultry, a different approach compared to traditionally morphological and functional observations could be helpful. One of the approaches is to study gene expression with a genome-wide array at different time intervals during development. With microarray analysis, the expression of all genes of a tissue can be investigated. Subsequently genes can be attributed to certain developmental clusters based on the observed expression patterns. Moreover, by functional analyses of the genes in each cluster, it is possible to assign them to a specific developmental category.

To investigate organisms with poorly annotated genomes, like poultry, with this approach is a challenge. Chicken microarray gene expression could be more

difficult compared to mice or rats, because only one third of the chicken probes map back to stable gene identifiers. Also the chicken biochemical and signaling pathways are poorly annotated, fewer pathways are described and the pathways describe fewer genes compared to the human pathways. Therefore, human orthologous in a human background were used for the various functional analyses. A disadvantage is that chicken specific genes and processes could not be studied with this approach.
The objective of this study was to describe chicken jejunal development by investigating gene expression patterns in time. This approach has the potential to assign putative functions of biological processes underlying development. We hypothesize that gene expression patterns run parallel to immunological, morphological, and functional developments as measured by traditional methods.

2.2 Material and Methods

Experimental design

Broilers (Ross 308) were housed in ground cages and had access to feed and water ad libitum. At seven time points, 8 h, 1 day, 2, 4, 8, 12 and 21 days post-hatch, 10 randomly selected chicks were killed by cervical dislocation. From these chickens body weights were measured and when no abnormalities were observed, jejunal sections were collected for RNA isolation. For the microarray analysis five jejunal samples of chickens with similar weights were chosen per time point to create a homogeneous group out of the 10 randomly chosen chicks. The study was approved by the institutional animal experiment committee, in accordance with the Dutch regulations on animal experiments.

RNA extraction

Frozen jejunum samples were homogenized in liquid nitrogen using a mortar and pestle. 50–100 mg of the homogenized tissue samples were dissolved in 1 ml of TRIzol reagent (Invitrogen, Breda, The Netherlands) using a syringe and 21-G needle passing the lysate for 10 times. After centrifugation the supernatant was transferred to a fresh tube and phase separation with chloroform was performed. The RNA was precipitated using 500 ml 2-propanol, and extra purification steps were performed with the Macherey-Nagel NucleoSpin1 RNA II kit (April 2007/Rev. 07). The purified end-product was used for microarray analysis. With the Agilent Bioanalyzer (lab on chip, Agilent, Santa Clara, USA) the quality and integrity of the RNA samples was analyzed.

Labeling, hybridizations, scans and feature extraction

The Agilent 4x44K chicken arrays (AMADID 015068) contain 43,451 probes, including internal controls, were used for hybridization. All probes were synthesized on the glass slide. One sample was hybridized on the array using a single dye hybridization approach, to synthesize Cy3 labeled cRNA the Agilent Low-Input Labeling Kit was used. From each RNA sample 500 ng was used for labeling. Afterwards the concentration and incorporation of the cRNA and dye were measured using the Nanodrop. For the hybridization 850 ng Cy3 labeled cRNA was used for further fragmentation and hybridizations. The Agilent protocols (GE1-v5_95_Feb07) were strictly followed for the hybridizations, washing, staining and scanning procedures. All hybridizations were performed in a controlled environment and one batch of dye was used to decrease the possibilities in variation between arrays.

The extended dynamic range (XDR) function was used to extend the dynamic range with 10-fold for the scanner. For this function, the arrays were scanned twice with 10% PMT and 100% PMT laser power. The Feature Extraction Software version 9.5, POTOCOL ge2_V5_95 from Agilent was used to generate the feature extraction data. For the background subtraction the options 'No background subtraction' and 'spatial detrend' were used. No background subtraction is default in Agilent protocols, this option is only true when arrays have issues, like high local background. On our arrays no issues were identified and by subtracting the background more variation in low expressers is introduced [6] (Suppl. Table SX1 and Suppl. Fig. SX1; see online version). Spatial Detrend attempts to account for low signal background that is present on the feature ''foreground'' and varies across the microarray. For spatial detrending, Agilent separates the surface fit and subtraction processes that must exist together to remove the surface trend found in the data. The advantage of this method is that it can remove variation in foreground intensity for different regions of the microarray. By performing spatial detrending or subtracting the surface fit through this foreground, the data becomes more consistent and reproducible. Also a linear/loess normalization on singular spots is performed.

Data loading and normalization

The files generated by the feature extraction software were loaded in GeneSpring GX 9.0.5, in which a log2-transformation and quantile normalization were performed. Quantile normalization is useful for normalizing across a series of conditions. According to Bolstadt et al. [7], quantile normalization is performed by four subsequent steps. Step (1): creates a matrix *X*, with dimensions *p x n*, where *n*

is the number of arrays and *p* the number of genes. Step (2): sorts each column of *X*, resulting in the new matrix *Xsort1*. Step (3): takes the means across the rows of *Xsort1* and assigns this mean to each element in the row to get a new matrix *X_sort2*. Step (4): rearrange each column of *X_sort2* to the same ordering as the input matrix *X*, and this is *Xnormalized*. After quantile normalization, the median of each sample is zero and the variation is in the same range (Suppl. Fig. SX2; see online version). Moreover, the principle component analysis (PCA) shows that there are two 'outliers', but samples belonging to the same group (time point) are adjacent to each other (Suppl. Fig. SX3; see online version). Thus, our quality controls show that our data does not contain artifacts or other problems.

Statistical and cluster analyses

A Kruskal–Wallis test (non-parametric one-way ANOVA) was performed on the normalized data, comparisons were made between subsequent time points, and thus in total six comparisons were made. p-Values are calculated via permutation tests (10,000 rounds), no assumptions are made for the test metrics computed to follow a certain fixed distribution [8]. The samples (different arrays) were shuffled in every permutation round and a test metric was calculated. For a certain gene, the 'new' p-value was the fraction of permutations in which the test metric computed was larger in absolute value than the actual test metric for that gene. After the Kruskal–Wallis test a fold change (FC) was performed to filter for highly differentially expressed genes, but only one out of six comparisons must have a FC equal to two or higher. These two tests were sequentially executed and resulted in 2,239 significant probes. Because the option 'spatial detrending' was performed we can accurately calculate fold changes between time points (arrays).

With this subset of 2,239 probes the Self-Organizing Maps (SOM) clustering method [9] was performed. The Self-Organizing Maps clustering method [9] includes the arrangement of the clusters on a two-dimensional grid, where similar clusters are neighbors and dissimilar clusters are placed far apart in the grid. Thus, probes displaying similar expression patterns in time cluster and may be involved in similar developmental processes. This does not mean that genes with a different expression pattern cannot contribute to the same biological process. Nevertheless, linear cluster analyses are generally used as an exploratory instrument to identify genes involved in similar or related processes and to identify hitherto unknown pathways associated with a developmental process. The following settings were applied: a Euclidean distance metric for the clustering of the probes and 500 iterations. The grid was 3 columns by 3 rows resulting in nine clusters with a hexagonal topology. The initial learning rate was set at 0.03, the initial

neighborhood radius was set at 5 and the neighborhood type was bubble. Nine clusters were chosen arbitrarily because several patterns could be observed, not only up or down in time but also different varieties.

Functional analysis

Separate analyses for pathway (Suppl. Table S1; see online version) and gene ontology (Suppl. Table S2; see online version) enrichment were performed, but the number of genes from the study set that overlap with certain GO terms or pathways was very small. Therefore, the Database for Annotation, Visualization and Integrated Discovery (DAVID) website [10] was used, which can give more biological insight behind large gene lists. In the functional annotation clustering method, different data sources are combined and analyzed by heuristic fuzzy multiple linkage partitioning [11]. For every cluster separately a functional annotation clustering was performed. The inputs were lists with gene symbols and therefore both chicken and human analysis could be performed. Because human has a better annotation and more databases are available, all the clusters were analyzed by choosing a human background. For every functional grouping an enrichment score (ES) was calculated, by the following formula:

$$ES = -log\sqrt[n]{p_1 x p_2 x \dots x p_n}$$

where ES is the enrichment score, and p_1, p_2 and p_n are the individual p-values of the associated database term. Stringency was set at high, which implies that similarity term overlap was set to 3 and the corresponding threshold to 0.85. Furthermore, the initial group membership, as well as the final group membership were set to 3, and the multiple-linkage threshold was set to 0.50. The data sources and their corresponding databases used are summarized in Table 2.1. Performing the analysis with the aforementioned stringency on a gene list of interest, results in a number of functional groups. Each group can contain one or more terms from the included databases and based on these terms a general term for each group is created.

Table 2.1 Sources and corresponding databases used for analysis

Source	Databases
Gene Ontology	Biological Process_All levels
	Molecular Function_All levels
	Cellular Component_All levels
Protein Domains	INTERPRO
	PIR SUPERFAMILY
	SMART
Pathways	KEGG
	BBID
	BioCarta
Functional Categories	COG-ONTOLOGY
	UP SEQ FEATURE
	SP PIR KEYWORDS
Disease	OMIM disease

2.3 Results

Per microarray an individual chicken jejunal sample was hybridized and this enabled us to make comparisons across microarrays and between groups of samples. The variation within groups of individual chickens is small as visualized by hierarchical clustering on the samples (Suppl. Fig. S1; see online version), thus comparisons between time points are possible. The microarray slides contain 43,451 probes and in the first step 31,040 probes were excluded which were not significant when testing for differences between two successive time points by an ANOVA test. With the resulting 12,411 probes a second filtering step was performed: probes which had a fold change higher than two in one out of the six sequential comparisons of time points were included for further analysis. The resulting 2,239 probes were used for a Self-Organizing Maps clustering where a grid of 3x3 was used. This resulted in nine clusters which display different expression profiles over time (Fig. 2.1). All 2,239 probes are represented in the graphs, where every line represents a probe with its corresponding normalized intensity per time point. Note that negative expression values can be observed due to the quantile normalization procedure. The clusters show nine different expression patterns. Cluster 1 has a low–zero–low expression pattern, clusters 2 and 3 show a high expression at the start and low expression later in time. Also cluster 6 shows high expression in the beginning, but thereafter it fluctuates around zero. Clusters 5 and

9 show contrasting expression patterns, cluster 5 shows high expression at time points 1 and 12, whereas cluster 9 displays low expression. Clusters 7 and 8 show low expression at the start and steadily increasing expression at later time points. The probes in cluster 4 show relative low expression at 8 hpi, comparable to clusters 7 and 8, and close to zero expression later in time.

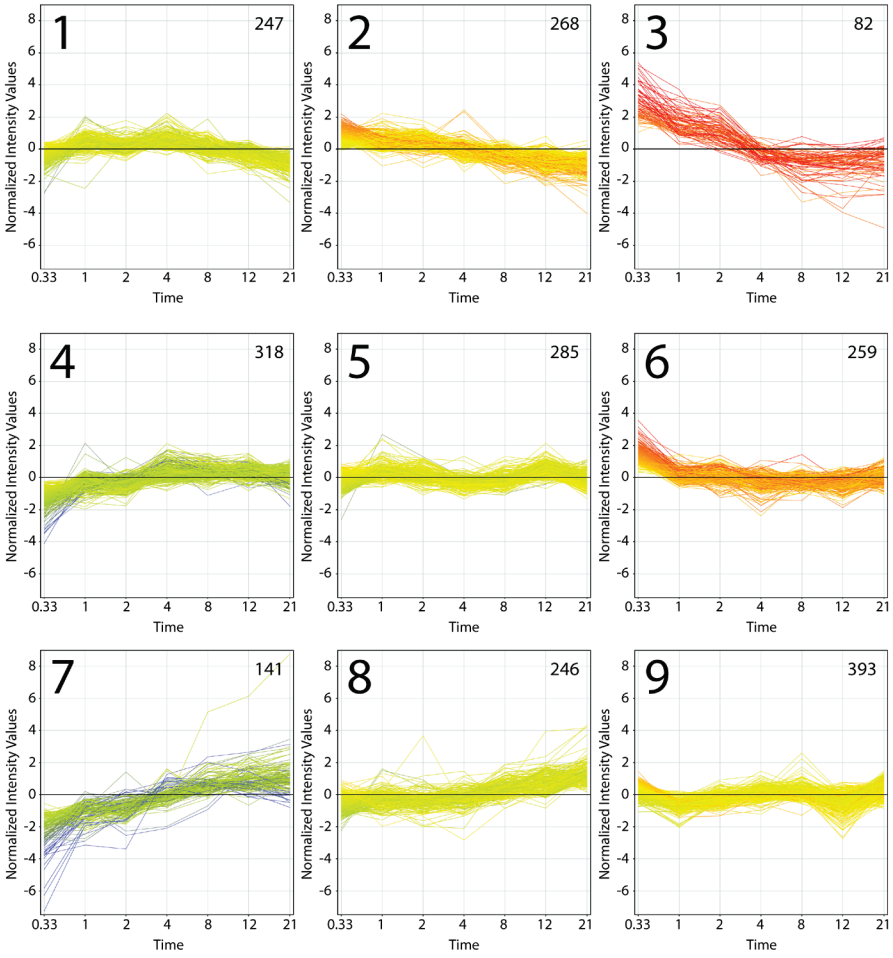

Figure 2.1 Graphical representation of probes of each cluster separately
The 2,239 probes, remaining after the statistical tests, are clustered into nine clusters. In the graphs on the y-axis the normalized intensity values are depicted, and on the x-axis the seven time points (days) are displayed. In each graph at the top-left the number of the cluster is depicted, and at the top-right the number of probes in the particular cluster.

After clustering, pathway and Gene Ontology analyses were performed on each cluster separately (Suppl. Tables 1 and 2; see online version). Due to the observed small numbers of enriched GO-terms or pathways, the functional annotation cluster method [11] is performed. The main difference is that the functional annotation clustering method uses more databases at once and combines the individual scores into a functional group enrichment score (ES). The number of genes that are involved in a functional group are higher than in the separate pathway and GO analyses.

All clusters had more than one functional group assigned, these functional groups were sorted on ES per cluster. The resulting top three functional groups of every cluster (Table 2.2) were similar to the top hits of either 'normal' pathway or GO analyses. For the analysis against chicken databases (Suppl. Table 3; see online version) the ES were overall lower compared to the analysis using human, but high ranking terms were similarly represented in both analyses.

Table 2.2 DAVID Functional Annotation Clustering [10, 11]

Cluster	TOP	General Term for Functional Group	ES	Study Set	Number of Genes Involved
1	1	Cell cycle	8.63	78	14
	2	Chromosome	4.28		6
	3	Reproduction / gamete generation	4.16		10
2	1	Ankyrins	1.43	52	4
	2	Reproduction / gamete generation	1.36		4
	3	(neuron) Cell morphogenesis	1.1		4
3	1	Metabolic process	3.22	24	6
	2	Metabolic process (acids)	2.98		6
	3	Transporter activity	0.63		5
4	1	Induction apoptosis	2.79	99	7
	2	Regulation apoptosis	1.89		12
	3	(innate) Immunity	1.8		6
5	1	Adhesion	0.66	39	6
	2	Development (general)	0.64		7
	3	Lumen	0.59		5

6	1	Kinase activity	2.31	85	3
	2	Nucleotide metabolic process	1.5		5
	3	Metabolic process	1.31		4
7	1	Signal / receptor activity	6.29	51	22
	2	Immune system development	3.02		5
	3	Scavenger receptor	2.51		3
8	1	Plasma membrane	5.35	76	21
	2	Response (defense)	4.49		16
	3	Signal / receptor activity	3.69		23
9	1	Biosynthesis	2.16	125	5
	2	Organelle / intracellular membrane	1.79		66
	3	Response (toxin / xenobiotic)	1.68		3

Abbreviations: ES, enrichment score.

In cluster 1, displaying a low-zero-low expression pattern, the following terms were identified: cell cycle, chromosome and reproduction/gamete generation. Clusters 2 and 3, with high expression first and a decreasing expression later in time, show no obvious functional grouping that corresponds to jejunal development. In cluster 3 only metabolic processes are present, whereas in cluster 2 functional groups as ankyrins and (neuron) cell morphogenesis are present. The pattern of cluster 4 is first low expression and then expression is close to zero, here the observed terms are related to induction, regulation of apoptosis, and innate immunity. The top three of cluster 5 consists of adhesion, development, and lumen, but all ES are below 1. Cluster 6 is partly homologous to clusters 2 and 3 especially at the early time points; the identified functional groupings can be summarized as metabolic related processes. Low expression at hatch and increasing expression over time. Clusters 7 and 8, both have the functional group signal/receptor activity. Cluster 7 has two other terms that are immune related, immune system development and scavenger receptor. Moreover, cluster 8 shows the terms (defense) response and plasma membrane, however approximately fifty percent of the genes in the term plasma membrane are immune related genes. In cluster 9 more metabolic related terms are represented, while the functional group organelle/intracellular membrane has halve of the genes involved in this particular term.

2.4 Discussion

Here we provide evidence that microarray gene expression studies, followed by cluster analysis and subsequent functional analysis on each individual cluster contributes to the discovery of specific details of developmental processes, even though the chicken genome is poorly annotated. This proposition is based on our observation that the expression pattern of a number of gene sets runs in parallel to several immunological, morphological, and functional developments as measured by other, more traditional methods, like immunohistochemistry, cell-counting and histological observations. Functional annotation clustering analyses showed that clusters with a high expression immediately after hatch and decreasing expressions later on, display several features of morphological and functional development. In contrast, clusters with low expression at hatch and increasing expressions up to 21 days, display features related to immunological development. These expression patterns coincide with what we know about the timing of jejunal development based on previous observations [2,4]. Due to the fact that we wanted to study intestinal development and not the gene expression of single cell subtypes the whole intestine was used to study gene expression. Thus, whole tissue samples were used, consisting of several cell types, and therefore we provide evidence of specific processes involved in jejunal development. The developmental processes could be linked to certain cell types, apoptosis and turn-over are more likely to be involved with epithelial cells. Whereas immunological development reflects the different immunological processes due to different cells present in the jejunum, for example intraepithelial lymphocytes.

In cluster 1, the normalized intensity values are first low, then around zero and finally low again. Functional annotation clustering analysis showed that in this cluster the cell cycle process is highly enriched, as well as the functional group chromosome. Indeed from literature it is known that during the morphological development of the intestine the plateau for crypt numbers is reached 2–3 dph [1], and between 1 and 4 days we measured the highest normalized intensity values. This provides evidence that at least a subset of the genes in cluster 1 is associated with cell division, cell morphology and contributes to villus growth. The presence of the functional group reproduction/gamete generation in clusters 1 and 2 is surprising. However, taking a closer look at the genes of this functional group, we observed that these genes are also involved in other processes like metastasis of cancer, due to the incomplete annotation of genes. Thus, it is important to put the findings into a biological perspective.

It is hypothesized that genes important for (the onset of) morphological and functional developments have the highest expression levels early in life. In that aspect the genes present in clusters 2, 3 and 6 (discussed later) are of interest, because they show a high expression at the start and low expressions later in time. The onset of morphological and functional development in the gut occurs at the embryonic stage. Genes important for intestinal development are involved in the hedgehog and WNT signaling pathway, moreover an important group of transcription factors are the homeobox (HOX) domain containing genes [12,13]. In total ten genes involved in these intestinal development processes are scattered over four clusters, namely 2, 3, 6 and 9.

In cluster 2 only one of these genes is present and is involved in WNT signaling, a similar result is observed in cluster 3 only here the gene is involved in hedgehog signaling. In cluster 2 the top functional groups are ankyrins, reproduction/gamete generation and (neuron) cell morphogenesis. Ankyrins link integral membrane proteins to the underlying cytoskeleton and are involved in a variety of activities such as cell proliferation, contact, activation, motility, and the maintenance of specialized membrane domains. This ankyrin motif is identified in many proteins, such as cyclin-dependent kinase (CDK) inhibitors, developmental regulators, cytoskeletal organizers and transcriptional regulators [14,15]. The (neuron) cell morphogenesis contains processes which are involved in generating and organizing anatomical structures. It is plausible that these genes (THBS4, CHL1, MT3 and TGFB2) are involved in neuron development, because nerve endings are also present in jejunal tissue. In cluster 3, two functional groups related to metabolic processes are represented and also the term transporter activity is present. It is possible that immediately after hatch and the first feed intake, genes involved in metabolic processes need to be switched on. This initial differential expression might be necessary to start the different metabolic processes. Thus, the genes in these functional groups could be of importance in chicken jejunal development, the observed terms in this analysis give evidence in the direction of both morphological and functional development.

Cluster 4 has a characteristic pattern, consisting of low expression at the first two time points and later in time normalized intensities around zero. The functional annotation clustering method formed the following groups; induction of apoptosis, regulation of apoptosis, and (innate) immunity. This suggests that during early time points there is less activity of apoptosis and (innate) immunity. It is plausible that this occurs in jejunal development, because apoptosis is the end process of cell (enterocyte) differentiation. It was found that only 2 days post hatch rapid morphological changes occurs [16], indicating that cell differentiation has been

started and therefore it could be that genes involved in apoptosis are not expressed earlier in time. The cluster 4 genes involved in (innate) immunity, are low expressed early in life. The genes present in the innate immunity group were C1S, S100B, C1R, C1QC, DMBT1 and F3. All genes are involved in immune related processes, and a subset of these six genes are involved in different processes related to the complement activation and regulation of inflammation. C1QC, C1R and C1S, which have similar gene expression patterns, form an antibody antigen complex in the classical complement pathway [17,18]. The observed expression pattern possibly corresponds to feeding behavior, if feed enters the jejunum local innate responses may be triggered. Another possibility is that bacterial colonization occurs after hatch, however chickens were raised in a controlled environment in this study.

Cluster 5 has high expression at days 1 and 12, and at other time points the normalized intensity is around zero. Low enrichment scores of below one are observed for all functional groups, the top three consisting of adhesion, development and lumen. This implies that the underlying terms from several databases have a low significance score. When performing separate pathway and GO analysis also low significant scores are observed. Apparently this cluster has a peculiar gene expression pattern in time and could not be coupled to specific developmental hallmarks.

Cluster 6, has comparable high expression early in time as clusters 2 and 3, encompasses genes which are involved in metabolic related terms. Kinase activity, nucleotide metabolic process and general metabolic process are identified as top hits. The expression is high 8 h post-hatch, and similar to cluster 3 metabolic related processes are observed. This strengthens that genes involved in metabolic related processes are high expressed early in life and either will go to a steady level or decrease in time. The metabolic related processes found in clusters 3 and 6 indicate functional development in the jejunum. Also two genes involved in hedgehog and WNT signaling are present which are important for morphological development.

Clusters displaying low expression early in time and increasing expression later are potentially involved with the (developing) immune system (clusters 7 and 8). After hatch chickens begin to eat, feed enters the intestine and trigger immune maturation. The direct immune response is mostly orchestrated by the innate system, and adaptive responses emerge later. In our study the gene expression of clusters 7 and 8 is above the normalized intensity of zero, from days 4 till 21 and is increasing over time. In cluster 7 the signal/receptor activity group, immune system development and scavenger receptors are present. Many of the genes (>60%)

involved in signal/receptor activity are related to immunity, and have a tendency towards adaptive immunity. The gene expression pattern of the functional group of immune system development corresponds to immune cell activation is initiated as early as 4 days post-hatch by cytokine gene expression (CD3gd, IFNγ, Bu-1 and IL-2) [4]. Also the presence of the scavenger receptor group in cluster 7 points towards the induction of immune functions, although scavenger receptors are known to be involved in innate immunity [19] and are mainly expressed on macrophages. This suggests that besides adaptive immunity also parts of innate immunity are present in this cluster. Cluster 8 also displays evidence of immune related processes. The following terms are observed: plasma membrane (defense) response and signal/receptor activity. Most of the genes involved in plasma membrane are immune related, which play a role in processes like adaptive immunity or cell adhesion. The term (defense) response is a summary of wounding, inflammatory, external stimuli and stress response. The same group as in cluster 7 is represented in cluster 8, the signal/receptor activity. Here also approximately fifty percent of the involved genes relate to immune related processes. Thus, clusters 7 and 8 can be associated with immunological development. The normalized intensity in these clusters 7 and 8 is above zero from around days 4 till 21. Clusters displaying a low to high expression pattern over time, contain genes which are involved in immunological processes.

Cluster 9 has in general an opposite expression pattern to that of cluster 5 and harbors functional groups involved in biosynthesis organelle/intracellular membrane and (toxin/xenobiotic) response. Here biosynthesis is mainly the production of steroids, sterols and lipids. Where three out of the five genes involved in the biosynthesis group are related to cholesterol synthesis, which is possible because it was already observed that de novo cholesterol can be produced in the distal jejunum and occurs at a low rate [20,21]. But it is difficult to link these processes to the particular pattern of cluster 9. More than fifty percent of the genes of the study set are involved in the organelle/intracellular membrane group, but all underlying evidence results from general terms. It is not possible to link the groups of genes to certain more specific processes. The group (toxin/xenobiotic) response consists of only three genes and is involved in the response to foreign substances entering the intestine. It is difficult to interpret the gene expression pattern over time of cluster 9. It might be that the observed immunological related processes are due to the increase expression between days 12 and 21. When analyzing genes separately, three genes are observed which play a role in the WNT signaling pathway. The identified functional groups could be involved in intestinal

developmental processes, evidence of morphological, functional and immunological development are observed.

The analysis of the gene expression data at the level of clusters and functional groups as discussed above, provides a global insight into the onset and timing of various biological processes. However, not all genes that are represented within one of the clusters are grouped by DAVID functional annotation clustering. When we reanalyzed the individual genes within one cluster by hand, we were able to extract more detailed information from the cluster datasets. As an example, we analyzed the clusters where immunologic related functional groups were detected. Within clusters 4, 7, and 8 the individual genes were analyzed (Suppl. Table 4; see online version) and grouped (Table 2.3).

Table 2.3 Overview of genes and immunological functions of genes present in clusters 4, 7, and 8

Cluster numbers, immunological functions, and relevant genes related to the particular functions are indicated

C	Immunological Function	Genes	References
4	Innate	C1R[a]+C1S[a]+C1QC[a]+F3[b]+S100B[c]+MST1[d]+ LRRC31[e]+LRRFIP1[e]+TRAF3IP2[f]+LOC427406[g]	[a][22][b][23][c][24][d][25][e][26][f][27][g][28, 29]
	T-cell / NK cell	CD2	[30]
	Migration / Adhesion	CD44	[31]
	T-cell: Activation	BCL2A1	[32]
	T-cell: Development	CD274[h]+ SOCS1[i]+SOCS3[i]	[h][33][i][34]
	T-cell: Proliferation	CD274[h] +FAS[j]	[j][35]
	Mucosal defense system	DMBT1[k]+MUC22[l]	[k][36][l][37]
	Cytokines / Chemokines	SOCS1[i] +SOCS3[i] +CCR8[m]+LOC427406[g]	[m][38]
7	MHC class I or II	B-LA[a]+B-MA2[a]+B2M[b]+CD74[c]	[a][39, 40][b] [41][c][42]
	B-cell	BANK1[d]+BTK[e]+ZAP70[f]+SPI1[g]	[d][43][e][44]f[45][g][46]
	T-cell: Activation	CD28[h]+CD8B[i]+CD74[c]+IL7[j]+ZAP70[k]+LCP2[l]	[h][47][i][48] [j][49][k][50-52][l][53-55]
	T-cell: Development	CD28[h]+CD8B[i]+IL12RB2[m]+HAVCR1[n]+PTPN22[o]+ IL7[j]+ZAP70[k]+LCP2[l] +STAT4[p]	[m][56][n][57][o][58][p][59-61]

	T-cell: Proliferation	CD28[g] +IL12RB2[l] +IL7[j]	
	Mucosal defense system	DMBT1[j]	
	Cytokines / Chemokines	CCRL1[q]+CSFR1[r]+IL7[j]+LECT2[s]	[q][62][r][63][s][64]
	Receptor / Recognition	IGJ[t]+CD200R1[u]+GZMA[v]+PIGR[w]+MARCO[x]+ LTB4R[y]+IL12RB2[m]+SCARA5[z]+SCARB1[z]	[t][65][u][66][v][67][w][68][x][69][y][70][z][71, 72]
	Bactericidal	BPI[aa]+RFSR[bb]+MARCO[x]	[aa][73][bb][74]
	Innate	C7[a]+LOC418832[b]+AvBD2[c]+AvBD7[c]	[a][75, 76][b][c][77]
	B-cell	LY86[d]+CD79B[e]+POU2AF1[e]+IL1R2[f]	[d][78][e][79][f][80]
	T-cell: Activation	CD8A[g]+IL1R2[f]	[g][81, 82]
	T-cell: Development	CD8A[g]	
8	Cytokines / Chemokines	IL1R2[f]+IL8RB[h]+CCR2[i]	[h][83][i][84]
	Receptor / Recognition	CD163[j]+TLR2[k]+LOC418412[l]+LOC416633[m] +IGSF6[n]+IL1R2[f]+IL8RB[h]	[j][85][k][86][l][87][m][88][n][89]
	Response	ACHE[o]+ADAMTS13[o]+EDN1[o]+SOD3[o]+ STAB1[o]+ TULP1[o]+C7[a]+LY86[d]+LYZ[o]+IL8RB[h]+TLR2[p]+CD8A[g]	[o] DAVID tool [10, 11] [p][90]

Abbreviations: C; cluster.

When investigating these immunological functions in the different clusters in relation to the observed gene expression patterns, several interesting aspects of immunological development can be observed. In cluster 4, with low expressions at the first two time points and normalized intensities around zero later in time, the main gene functions are related to the process of cell migration and to the development of innate immunity. Whereas the genes present in cluster 7, displaying low expression early in time and increasing expression later, are mainly involved in differentiation and specialization of immune cells. In cluster 8, with normalized expression levels around zero in the beginning and increasing expression later on, many genes are present with functions necessary for the regulation and development of immune responses. These spatio-temporal patterns of gene expression seem to be logical because influx of immune cells is necessary prior to immune differentiation. In addition, it is also logical that immune regulation is required as soon as immune cells mature.

Therefore, our whole genome approach followed by SOM and functional annotation clustering methods, provides clues to get more detailed insights in the

various aspects of jejunal development. We have shown that the spatio-temporal immunological development of the chicken intestine can be split into at least three different functional events. Apparently, first innate immune development and immigration of immunological cells occurs. Thereafter a process of immune differentiation and specialization occurs, whereas later in time maturation and immune regulation seems to be important. With the functional analyses at the gene level, we could not demonstrate a spatiotemporal separation between the development of the innate and adaptive branches of the immune system, in contrast these processes seem to be intertwined, at least in the chicken intestine.

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3

Effects of *Salmonella* on Spatial-temporal Processes of Jejunal Development in Chickens

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Abstract

To study effects of *Salmonella enteritidis* on morphological and functional changes in chicken jejunal development, we analyzed gene expression profiles at seven points post-infection in 1–21 day-old broiler chickens. Nine clusters with different gene expression patterns were identified, and the genes in each cluster were further analyzed by a functional annotation clustering method (DAVID). Functional and morphological developmental processes dominated in all the nine clusters. *Salmonella* infection caused delays in several intestinal-morphological processes, whereas functional metabolic processes occurred in a similar spatial-temporal frame compared to normal jejunum development. A clear difference between normal developing- and *Salmonella* disturbed jejunum was the higher expression of genes involved in cell turn-over at early stages in the infected jejunum. Surprisingly, we found no clustered immune related processes in the infected birds. To compare the immunological processes between control and *Salmonella* infected chickens, the gene expression data was superimposed on known immunological KEGG pathways. Furthermore an in-depth analysis on the immune gene level was performed. As expected, we did find immunological processes in the *Salmonella* infected jejunum. Several of these processes could be verified by immunohistochemistry measurements of different immunological cell types. However, the well-ordered spatial-temporal development of the immune system, as observed in control non-infected animals, was completely abolished in the infected animals. Several immunological processes started much earlier in time, whereas other processes are disorganized. These data indicate that normal morphological and immunological development of jejunum is changed dramatically by a disturbance due to *Salmonella* infection. Due to the disturbance, the well-organized spatial-temporal development of morphological processes are delayed, those of the immunological development are scattered, whereas metabolic functional processes are almost not affected. This demonstrates the flexibility of developmental processes in the broiler chicken intestine.

Key words: Microarray, Time-series, Intestinal development, DAVID analysis, KEGG analysis

3.1 Introduction

The chicken jejunum rapidly develops after birth. Morphologically, its length increases rapidly as well as the height of the villi, and also the function of the jejunum changes rapidly after birth as food absorption starts. In early life major immunological developments occur in the intestine. Luminal antigens and nutrition drive the expansion and differentiation of intestinal epithelial and lymphoid tissues in regulating immune responses to environmental antigens. Development of the chicken intestinal tract occurs in a well time-ordered sequence of spatial patterns and processes that can be divided into functional, morphological and immunological categories. Concomitantly, proteins and genes which are of importance within these categories have different spatial-temporal expression patterns [1-4]. In chickens, the genes involved in morphological development of jejunum are highly expressed immediately after hatch, whereas genes involved in immunological development and processes become more active at 4 days post hatch [5]. Most of the developmental studies in chickens are performed with healthy animals, and little is known of the quantitative and qualitative effects of disturbances on global jejunal development. Pathological and immunological effects of disturbances with infectious agents, as well as the effects of feed withdrawal and stress have been described for chicken, but these studies usually focus on specific processes [6-9]. For example, the jejunum in fasting chickens showed reduced levels of enterocyte proliferation and migration, and diminished crypt and villus development [6, 8], whereas simultaneously an increased colonization of pathogens occurred [10-12]. Moreover, it has been observed that stress may affect intestinal (barrier) functions [13-17] and disturbs nutrient absorption [18-21]. Spatial-temporal processes could be roughly divided in three categories, functional (metabolic), morphological and immunological, which all have specific patterns in regular intestinal development.

The objective of the present study was to identify the effects of a severe disturbance on normal jejunal development in chicken at a global scale gene expression level. Knowledge of the disturbance mechanisms may contribute to develop strategies to prevent the effects of intestinal disturbances. We investigated changes in chicken jejunal development at the gene expression level due to an infection with a pathogen and we focused on the time-ordered sequence of gene expression patterns and processes. We used *Salmonella enteritidis* as a severe disturbing factor since in the chosen infection model it enters the systemic system by transmigration of the intestine and induces clear clinical effects. The array data was verified by independent immunohistochemistry measurements.

3.2 Material and Methods

Experimental design

Chicks (Ross 308, broilers) were housed in ground wire cages with ad libitum access to feed and water. Animals were orally inoculated at day zero with 0.2 ml phosphate buffered saline (PBS) containing 1·10^5 *Salmonella enteritidis* (nalidixic acid resistant strain of *Salmonella enterica* serotype Enteritidis PT4 isolated from chicken [22]) or only PBS (as described by Schokker et al. [5]). At seven time points after infection (8 hrs and 1, 2, 4, 8, 12 and 21 days), 10 randomly selected chicks were killed by cervical dislocation. From these chicks, body weights were measured, liver and caecum were collected for determination of counts of *Salmonella enteritidis*. Jejunal sections were collected for RNA isolation. During the experimental period the chickens were daily observed in order to monitor their overall health. Signs of ruffled feathers and depression were taken as measurements for sickness. The study was approved by the institutional animal experiment committee, in accordance with the Dutch regulations on animal experiments. For microarray analysis, five individual RNA samples were prepared from a homogenous subgroup of individual chickens with similar weights were selected.

Bacterial Colonization

To detect *Salmonella* in the liver and caecum, 1 g of liver or caecal content was homogenized in 9 ml PBS and serially diluted in PBS of all 10 randomly selected chickens. The diluted homogenates were plated onto square Brilliant Green Agar (with 100 µg/ml nalidixic acid, BGA-Nal+) and after 24 hours at 37°C colonies were counted according to the track dilution method described by Jett [23].

Microarray analysis

RNA isolation

Frozen jejunum samples were homogenized in liquid nitrogen using a mortar and pestle. 50–100 mg of the homogenized tissue samples were dissolved in 1 ml of TRIzol reagent (Invitrogen, Breda, The Netherlands) according to Schokker et al. [5]. An extra purification steps were performed with the Macherey-Nagel NucleoSpin® RNA II kit (April 2007/Rev. 07). The purified end-product was used for microarray analysis. With the Agilent Bioanalyzer (lab on chip, Agilent, Santa Clara, USA) the quality and integrity of the RNA samples was analyzed.

Labeling, hybridization, scans and feature extraction

The Agilent 4x44K chicken arrays (AMADID 015068) contain 43,451 probes, including internal controls, were used for hybridization. The array design file is available at ArrayExpress [24] (www.ebi.ac.uk/arrayexpress), with a unique identifier A-MEXP-1509. All probes were synthesized on the glass slide as was described by Schokker *et al.* [5]. All hybridizations were performed in a controlled environment and one batch of dye was used to decrease the possibilities in variation between arrays.

Furthermore the Feature Extraction Software version 9.5, PROTOCOL ge2_V5_95 from Agilent was used to generate the feature extraction data. For the background subtraction the options 'No background subtraction' and 'spatial detrend' were used, as described in Schokker *et al.* [5]. No background subtraction is default in Agilent protocols, this option is only true when arrays have issues, like high local background. On our arrays no issues were identified and by subtracting the background more variation in low expressers is introduced [25]. By performing spatial detrending or subtracting the surface fit through this foreground, the data becomes more consistent and reproducible. Also a linear/loess normalization on singular spots is performed.

Data loading and normalization

The files generated by the feature extraction software were loaded in GeneSpring GX 9.0.5, in which a log2-transformation and quantile normalization was performed. Quantile normalization [26] is useful for normalizing across a series of conditions. After quantile normalization, the median of each sample is zero and the variation is in the same range (Suppl. Fig. S1; see online version).

Statistical and cluster analyses

A Kruskal–Wallis test (non-parametric one-way ANOVA) was performed on the normalized data, comparisons were made between subsequent time points, and thus in total six comparisons were made. P-values are calculated via permutation tests (10,000 rounds), no assumptions are made for the test metrics computed to follow a certain fixed distribution [27]. After the Kruskal–Wallis test a fold change (FC) was performed to filter for highly differentially expressed genes, but at least one out of six comparisons must have a FC equal to two or higher. These two tests were sequentially executed and resulted in 5,136 significant probes. Because the option 'spatial detrending' was performed we can accurately calculate fold changes between time points (arrays).

With this subset of 5,136 probes the Self-Organizing Maps (SOM) clustering method was performed [28]. The different settings were similar as described in our earlier article [5]. The raw data, as well as the normalized data, are available at ArrayExpress [24] (www.ebi.ac.uk/arrayexpress) with unique accession code E-MEXP-2042. Splitting of the data, in control and *Salmonella* infected chickens, was performed to enrich for genes which have a time-effect. By normalizing both datasets separately, genes which are different between both groups can be discarded.

Functional annotation clustering

The Database for Annotation, Visualization and Integrated Discovery 2008 (DAVID 2008) [29] was used for functional annotation clustering. Within DAVID different data sources are combined and analyzed by heuristic fuzzy multiple-linkage partitioning [30]. For the genes present in every cluster a separate functional annotation clustering was performed (November 19, 2009). The inputs were lists with chicken gene symbols or the homologous human symbols. Because human has a better annotation and more databases are available, all the clusters were analyzed by choosing a human background. Moreover the settings in DAVID were similar as described by Schokker *et al.* [5]. Summarized in Table 3.1 are the data sources used with their corresponding databases.

Table 3.1 Sources and corresponding databases used for analysis

Source	Databases
Gene Ontology	Biological Process_All levels
	Molecular Function_All levels
	Cellular Component_All levels
Protein Domains	INTERPRO
	PIR SUPERFAMILY
	SMART
Pathways	KEGG
	BBID
	BioCarta
Functional Categories	UP SEQ FEATURE
	SP PIR KEYWORDS
Disease	OMIM disease

Immunological pathways

The following pathways were extracted from the KEGG database [31-35]: complement and coagulation cascades, toll-like receptor signaling pathway, natural killer cell mediated cytotoxicity, antigen processing and presentation, T cell receptor signaling pathway, B cell receptor signaling pathway and leukocyte transendothelial migration (Table 3.2). For each gene or gene complex the expression was checked, if it was below or above zero. Subsequently for each pathway the percentage of genes below or above zero is calculated.

Table 3.2 KEGG immunological pathways

Pathway	# of genes*	# of genes* on array	Immune component
Complement and coagulation cascades	58	40	Innate
Toll-like receptor signaling pathway	28	20	Innate
Natural killer cell mediated cytotoxicity	66	52	Innate
Antigen processing and presentation	72	34	Adaptive
T cell receptor signaling pathway	65	52	Adaptive
B cell receptor signaling pathway	37	28	Adaptive
Leukocyte transendothelial migration	56	45	Innate / Adaptive

*genes or gene complexes

Immunohistochemistry

From each animal, jejunum sections (8 µm thick) were collected at 8hpi, 1, 2, 4, 8, 12 and 21 dpi, which were immunohistologically stained by an indirect immunoperoxidase method. The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After being dried, the slides were treated with inhibitor for endogenous peroxidase for 5 min. The slides were subsequently incubated for 30 min with monoclonal antibodies against $CD8^+$ cells (1:200; CT-8, Southern Biotech), TCRαβ (1:50; TCR2, Southern Biotech) or TCRγδ (1:400; TCR1, Southern Biotech). This was followed by peroxidase-conjugated rabbit anti-mouse Ig (1:100; (P161) Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1M Tris–HCl solution (pH 7.5) containing 0.03% H2O2. The slides were further colored with 1% CoCl2 for 5min. After washing the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in distyrene-tricresyl phosphate-xylene (DPX). Images were acquired and

analyzed with Image-Pro Plus (version 6.2, media cybernetics). Per animal and staining three images were acquired of different regions. The average was calculated and divided by the area, resulting in the amount of cells per square millimeter of tissue.

3.3 Results

Bacterial colonization

For 10 chickens the *Salmonella* counts in liver and caecum were measured at 8 hours post-infection (hpi) until 21 days post-infection (dpi) (Fig. 3.1). In the liver of infected chickens up to 6×10ˆ3 colony forming units (CFU) per gram tissue were found at 1 dpi. Immediately after infection the *Salmonella* count is very high and then decreases slowly over time, reaching the zero level between 12 and 21 days. In the caecal content only at 1, 2 and 4 dpi bacteria were found, with a peak of 8.5×10ˆ8 CFUs at 4 dpi. The patterns of *Salmonella* counts in liver and caecum are depicted in Fig. 3.1. In control chickens no *Salmonella* was found at the different time points.

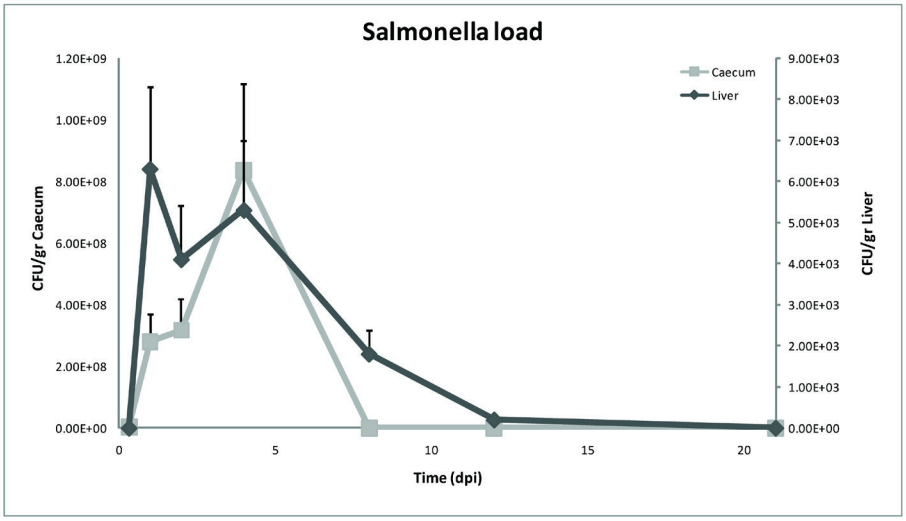

Figure 3.1 *Salmonella* counts in liver and caecum
On the x-axis the time in days post infection (dpi) is depicted and on the y-axis the Colony Forming Units (CFU) per gram tissue is depicted. The number of CFU *Salmonella* per gram of caecum (grey and primary axis) and liver (black and secondary axis). Per time point the value depicts the average of 10 chickens, also the standard error is given.

Microarray gene expression analysis

RNA, isolated from each individual chicken jejunal sample was hybridized to a microarray slide, which enabled us to make comparisons between the various experimental groups. All 43,451 probes on the array, with the exception of control probes, were included for the statistical analysis. After investigating differences between sequential time-points by an ANOVA test and the selection of probes with hybridization fold changes higher than two in one out of the six possible comparisons, 5,136 probes remained for cluster analysis. These probes were the input for clustering with the Self-Organizing Maps method. Application of the method resulted in the identification of nine different clusters in the *Salmonella* disturbed jejunum (Fig. 3.2) each showing a cluster-specific gene expression pattern.

Compared to normal intestinal development, clusters F, G, H and I show an irregularity at 2 dpi in their expression pattern. This irregularity suggests an association with the *Salmonella* colonization in the liver. Both clusters G, H, I and *Salmonella* colonization (liver) show a general increasing expression over time, with a dip in expression at 2 dpi. Cluster F, has an anti-correlation with the *Salmonella* colonization (liver), and thus a peak is observed at 2 dpi.

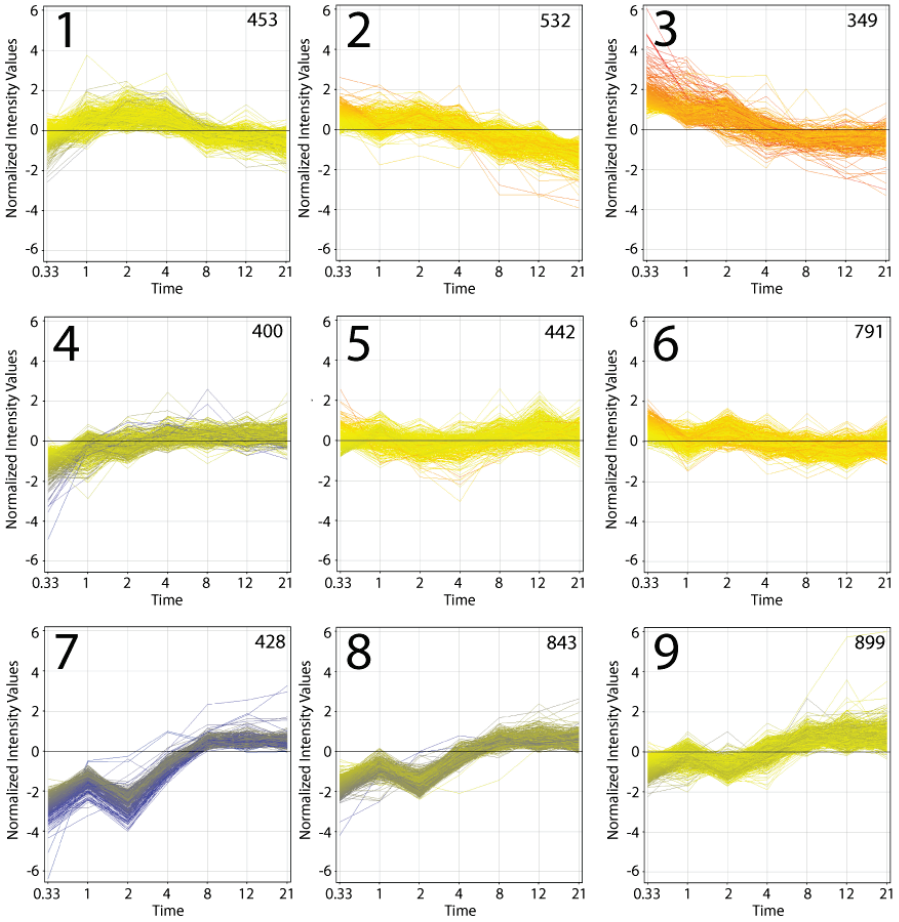

Figure 3.2 Graphical representation of the expression profiles in each cluster
On the y-axis the normalized intensity values are depicted, and on the x-axis the seven time points (days). In each graph at the top-left the cluster identifier is depicted, and at the top-right the number of probes residing in the cluster. Thus all nine graphs show a group of probes which have a similar expression pattern over time.

In cluster A low expression was observed at 8 hpi, high expression at 1, 2 and 4 dpi and low expression again at 8, 12 and 21 dpi. Both clusters B and C displayed patterns with high expression at the start and a decrease over time. Cluster D had first low expression, and a plateau around the normalized zero level was reached at 2 dpi. Expression in this cluster started below zero and increased expression is observed until day 2. From day 2 onwards expression was stable around zero. In cluster E expression was first above the normalized zero level and at 2, 4 and 8 dpi

below zero, then again above zero at 12 and 21 dpi. Cluster F showed high expression at 8 hpi and stabilized around zero with a little peak at 2 dpi. Clusters G, H and I had corresponding expression patterns, of which cluster G had the steepest slope. All the clusters G, H and I showed increasing expression over time and reaching a plateau around 8 dpi. In all the three clusters an irregular dip in the expression level is present at 2 dpi.

DAVID functional annotation clustering

`For each cluster depicted in Figure 3.2, a separate for functional annotation clustering analyses was performed with Database for Annotation, Visualization and Integrated Discovery (DAVID) [36], with default settings [36, 37]. The top 3 functional groups, based on Enrichment Score (ES), per cluster are listed in Table 3.3. In clusters A and B terms related to cell cycle processes were observed, as well as DNA damage and repair processes, and cluster A has very high ES for M-phase (cell cycle) process. The functional groups metabolic related processes and 'localization / transport' process pop-up in cluster C. Cluster D contains the terms ANK domain, plasma membrane and coagulation, with most of the genes belonging to the membrane functional group. The top three in cluster E consists of vitamin binding, metabolic process (nitrogen / amine) and oxido-reductase activity, but all functional groups have low numbers of genes. In cluster F the term thrombospondin is present twice and 57 genes within this cluster were found to be related to the term localization / transport. In cluster G fibronectin is observed as well developmental related terms. Cluster H, which displays a similar expression pattern to cluster G, contains the top three terms: cell migration/motility; cytokine biosynthetic process; and negative regulation of biosynthetic processes. In cluster I, the terms cell development/differentiation, extra cellular matrix and protein modification dominate.

The functional clustering results of this study were compared with the functional clustering results obtained during normal development of chicken jejunum using identical experimental conditions and methods for analysis [5]. The results of this comparison are presented in the discussion.

Table 3.3 Top 3 of Functional Annotation Clustering results

Cl	TOP	General Term for Functional Group	ES	Study Set	Number of Genes Involved
A	1	M-phase (cell cycle)	18.9	159	27
	2	Cytoskeleton	6.68		24
	3	DNA damage/repair	5.32		15
B	1	M-phase (cell cycle)	3.87	155	12
	2	Cell cycle process	3.19		16
	3	DNA damage/repair	2.27		9
C	1	Metabolic process (nitrogen / amine)	2.66	107	9
	2	Localization / Transport	2.14		29
	3	Metabolic process (nitrogen / amine)	1.25		3
D	1	ANK domain	1.97	114	6
	2	Plasma membrane	1.63		17
	3	Coagulation (blood)	1.61		4
E	1	Vitamin binding (pyridoxal phosphate)	2.65	132	6
	2	Metabolic process (nitrogen / amine)	2.53		10
	3	Oxidoreductase activity	2.35		4
F	1	Thrombospondin	2.84	257	6
	2	Localization / Transport	2.17		57
	3	Thrombospondin	2.04		5
G	1	Fibronectin	2.56	117	7
	2	Cell development / differentiation	2.41		24
	3	Development (general)	2		19
H	1	Cell migration / motility	2.64	223	13
	2	Cytokine biosynthetic process	2.11		5
	3	Negative regulation biosynthetic processes	1.91		4
I	1	Cell development / differentiation	3.02	249	39
	2	Extra cellular matrix	2.76		13

3	Protein modification	2.2	38

Abbreviations: Cl, cluster; ES, enrichment score; study set, number of genes in a cluster which maps to a stable gene identifier.

Immune related genes

In the DAVID functional annotation clustering analysis no immunological processes were identified in the *Salmonella* infected group. However in regular intestinal development, immunological related terms were observed, the corresponding clusters (4, 7, 8 and 9) contained high-ranked immune related functional groups indicating a well-ordered spatial-temporal development of immune related functions. Therefore, for every probe that maps back to a gene symbol in clusters D, G, H and I, its relationship to immunological functions was investigated. Also these genes were screened for associated GO-terms and/or involvement in immunological related pathways. Subsequently a literature investigation for immunological functions of these genes was performed. The resulting immunological related functional groups could be classified as either innate or adaptive immunity [5]. Furthermore adaptive immunity could be subdivided in either differentiation or regulation processes. Here we present a similar approach to broadly classify immunological related genes in clusters D, G, H and I of *Salmonella* infected chickens. An overview of the results is given in Table 3.4.

Table 3.4 In depth analysis of immunological enrichment in clusters D,* G,* H* and I*

C	Immunological Function	Genes	Ref.
D	Innate	A2M[a]; C1QA[b]; CD180[c]; DMBT1[d]; F8[e]; FGG[f]; LY96[c]	[a][31-34][b][38, 39][c][40][d][41][e][42, 43][f][44]
	Response	C1QA[b]; LY86[c]; CD180[c]; DMBT1[d]; F8[e]; FGG[f]; LTF[d]; FTH1[d]; TAPBP[d]; LTB4R[d]; AFAP1L2[d]; CXCL14[g]; ICOS[h] ; SYK[i]; CCR6[j]; PDCD1LG2[k]; HPSE[l]; FN1[m]	[g][45][h][46][i][47][j][48][k][49][l][50][m][51]
	Receptor / Recognition	IL13RA2[d]; CCR6[j]; GPR17[n]	[n][52]
	Wound healing	C1QA[b]; CD180[c]; LY86[c]; LTB4R[d]; PROZ[d]; MST1[d]; AFAP1L2[d]; F8[e]; FGG[f]; CXCL14[g]; HPSE[a]; FN1[m]	
	Cytokines / Chemokines	ASB3[o]	[53]
	T-cell: Activation	ICOS[h]; SYK[i]; PRLR[p]; SPI1[q]	[p][54][q][55, 56]
G	MHC class I or II	CD74[a]	[a][57]
	T-cell: Activation	CD74[a]; CBFB[b]; GZMA[c]; EPB42[d]	[b][58][c][59][d][60]
	T-cell: Development	LRMP[e]; IL9[f]	[e][61, 62][f][63]
	B-cell	LRMP[e]	
	Response	MBL2[g]; NFX1[h]; FN1[i]; ADRB2[j]; GABRA5[k]	[g][64][h][65][i][51][j][66][k][67, 68]
	Innate	MBL2[g]	
	Receptor / Recognition	PIGR[l]	[l][69]
	Cytokines / Chemokines	PIAS1[m] SEMA4D[n] TRIM9[o]	[m][70][n][71][o][72]
H	MHC class I or II	B-MA1[a]; B2M[b]	[a][73, 74][b][75]
	B-cell	IL5[c]; FCRL2[d]	[c][76, 77][d][78]
	T-cell: Activation	CD4[e]; LAT2[f]; LAG3[g]; IL10[h]; INS[i]; CD34[j]	[e][79, 80][f][81][g][82-84][h][85-87][i][88][j][89]
	T-cell: Development	CD4[e]; IL10[h]; TAL1[k]; TGFB2[l]	[k][90, 91][l][92]
	T-cell:	IL10[h]; TGFB2[l]	

	Regulation Cytokines / Chemokines	IL10[h]; IL5[c]; CSF2RA[m]	[m][31-34]
	Receptor / Recognition	IL2RG[n]; BN-K[o]; TPO[m]	[n][93, 94][o][95, 96]
	Response	S100B[p]; LAT2[f]; INS[i]; UBASH3A[q]; TGFB2[l]; GHSR[r]	[p][97][q][98] [r][41]
	Innate	LRRN1[s]; AvBD5[t]; PLG[u]	[s][99][t][100][u][31-34]
	Leukocyte transendothelial migration	CTNNA1[p] MYL9 [p]	
I	Antigen Processing and Presentation	B-LA[a]; B-MA2[a]; LOC417083[b]; CALR[c]	[a][73, 74][b][101][c] [31-34]
	B-cell	CD79B[d]; POU2AF1[e;] ; INPP5D[f]; RASGRP3[c]	[d][102][e][1 03][f][104]
	T-cell: Activation	CD8B[g]; GLMN[h]; SOD1[i]; FGB[j]; SFRS17A[k]; TNFRSF13B[l]; AGT[m]; SHH[n]	[g][105][h][10 6][i][107][j][1 08][k][109][l] [110][m][11 1][n][112, 113]
	T-cell: Development	CD8B[g]; CCL17[o]	[o][55, 56]
	T-cell: Proliferation	CD8B[g]; TNFRSF13B[l]; GLMN[h]; SOD1[i]; SHH[n]	
	Cytokines / Chemokines	TNFRSF9[p]; TNFRSF13B[l]; FLT3[c]; HGF[c]	[p][114]
	Receptor / Recognition	IGJ[q]; TRA@[r]; CCL17[o]; CD300L-S1[s]; IL2RG[t]	[q][115, 116][r][47][s] [117][t][93, 94]
	Bactericidal / Response	BPI[u]; LYZ[v]; ITGB6[w]; AKT3[x]; EGF[y]; FABP4[z]; FOS[f]; NDST1[o]; NFX1[o]	[u][118][v][119][w][120][x][1 21][y][122][z] [123]
	Innate	AvBD11[aa]	[aa][100]
	Wound healing	FN1[f]	
	Leukocyte transendothelial migration	CXADR[bb]; PTPRK[cc]	[bb][124][cc][125]

Abbreviations: C, Cluster; Ref., References. * The equivalent clusters in normal development displayed genes clustered as immunological functional groups

To view the spatial-temporal processes of immunological related genes in *Salmonella* infected chicken, gene expression data was superimposed on known KEGG immunological pathways. To this end, seven different pathways were extracted from KEGG [31-35]. All genes present on the microarray and present in a pathway were included with no restrictions on p-value, fold change or clustering. Moreover the gene expression of control and *Salmonella* infected chickens was superimposed, independently from the SOM clustering. For each pathway the number of genes the normalized expression below or above zero was determined and visualized (Fig. 3.3), resulting in a percentage (which is converted to the chosen color-scale). In the control situation all pathways exhibit an overall expression below zero before 4 days post hatch, whereas from 4 days post hatch an overall expression above zero is observed. This pattern is less clear in the *Salmonella* infected situation, where expression is already above zero at 2 days post infection in most cases and lesser genes are expressed above zero in late life compared to control chickens.

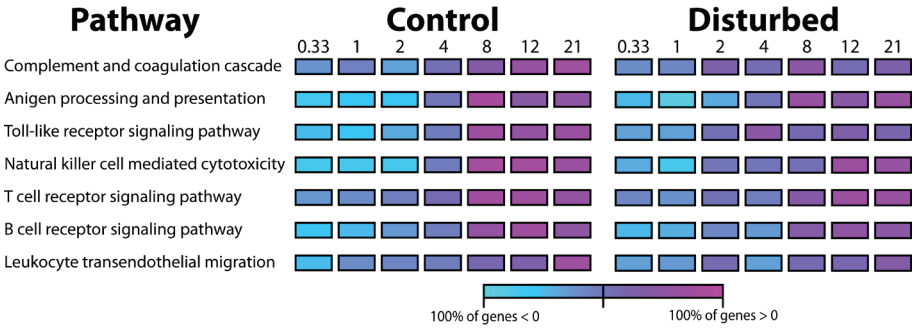

Figure 3.3 Overview of the overall expression of genes per pathway over time
Expression of genes involved in seven immunological pathways was investigated from 0.33 to 21 dpi in control or disturbed development. Every immunological pathway is separately analyzed. When all genes residing in the pathway are expressed below zero, the color is cyan. When all genes are expressed above zero, the color is magenta. In between is the color range from cyan to magenta; when 50% of the genes is expressed above zero and 50% below, the color is purple.

The antigen processing and presentation pathway and T cell receptor signaling pathway do not differ much between control and infected chickens; only in the antigen processing and presentation pathway at 8 dpi fewer genes are expressed above zero in infected chickens. In the complement and coagulation cascade as well as the Toll-like receptor signaling pathway genes are higher expressed at 2 dpi in *Salmonella* infected chicken compared to control chicken, and lower expressed at 12 and 21 dpi. The data shows that genes involved in the natural killer cell-

mediated cytotoxicity pathway in infected chickens are overall earlier expressed above zero compared to control chickens. In infected chickens the leukocyte transendothelial migration pathway and B cell receptor signaling pathway, all have a peak in overall expression of genes above zero at 2 dpi and fewer genes are expressed above zero at 4 dpi compared to control chickens. Thus the effect of *Salmonella* is shown by the overall expression of genes involved in immunological pathways, at 2 dpi already a difference in expression can be observed between control and infected chicken.

To validate the gene expression data, we measured the number of TCRαβ, TCRγδ and $CD8^+$ cells by immunohistochemistry (Fig. 3.4). The immune related data support the overall gene expression data and the conclusions drawn from this with regard to the TCR signaling pathway, as well as the natural killer cell mediated cytotoxicity (Fig. 3.3). The increase observed in the TCRαβ and TCRγδ in infected chickens is less smooth compared to control chickens. Also at 8 hpi in infected chickens, fewer positive cell counts were observed for both TCRαβ and TCRγδ in comparison to control chickens. No other significant differences were observed for TCRγδ, however for TCRαβ at 2 and 21 dpi significant difference between control and infected chicken was observed. This latter difference is not observed in the overall gene expression pattern of TCR signaling. In addition, when investigating the difference between infected and control chickens, significantly less $CD8^+$ cells are observed from 4 - 21 dpi in infected chickens, which strengthens our conclusion based on the gene expression data. In the first stage of life 0-2 dpi, similar $CD8^+$ cell counts are observed for both control and infected chickens.

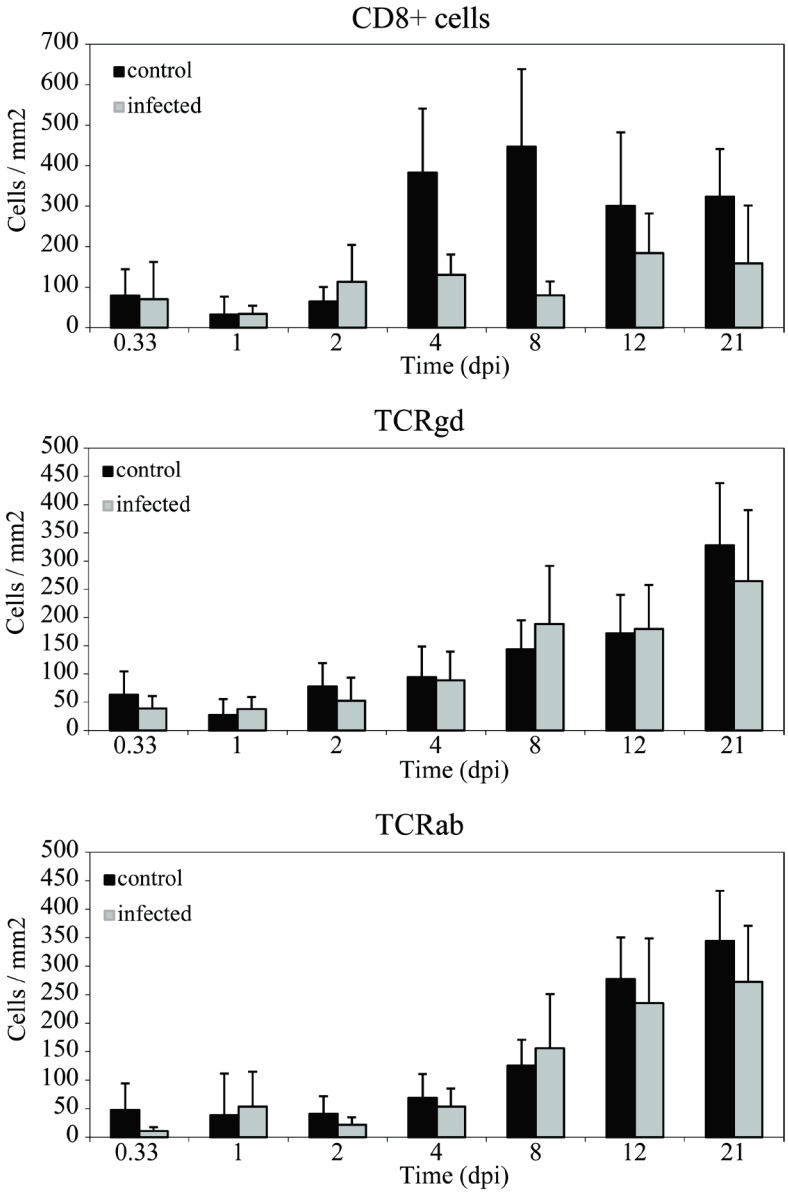

Figure 3.4 Bar charts of three immunological cell markers in control and *Salmonella* infected jejunum of chicken

Immunohistochemistry on jejunal tissue for $CD8^+$ cells (upper panel), TCRγδ (middle panel) and TCRαβ (lower panel), from 8 h post-infection to 21 days post-infection. Control chickens (black bars) and infected chickens (grey bars) are depicted, as well as the standard error at each time point.

3.4 Discussion

Bacterial colonization

The gene expression data presented in this paper indicate that the normal development of chicken jejunum, which is orchestrated into specific spatial-temporal patterns, is changed dramatically by a disturbance with *Salmonella*. Part of the data has been verified by independent immunological measurements. From the data it can be concluded that the effect of *Salmonella* during jejunal development is a delay of morphological processes of the jejunum, but an acceleration of cell turn-over processes. Striking is the effect of *Salmonella* on immunological related processes, which during normal development displayed well recognized and organized spatial-temporal patterns in time and which were not observed in the infected chicken. Even though the effect of *Salmonella* on jejunal development is rather harsh, developmental process in the jejunum did occur. This demonstrates that the jejunum developmental system is robust and able to develop via different routes and timelines, while immunological development is rather flexible.

Normal versus disturbed intestinal development

The focus of this study is to describe the change in gene expression patterns when comparing normal and *Salmonella* infected chicken jejunal development. When comparing the nine gene expression clusters of normal [5] and disturbed jejunal development, more or less similar expression patterns were obtained, probably due to the method of clustering. In clusters F-I of the disturbed analysis remarkable differences in the gene expression patterns related to disturbance were found that were absent during normal development [5]. Despite the overall similarity in gene expression patterns, completely different functional groups were found to be present in nearly all the clusters. Only in clusters 1 and 3 (normal jejunal development) compared to cluster A and C (disturbed jejunal development) similar functional groups were present. However other genes and other database terms, like GO-term or INTERPRO protein family were represented within these otherwise overlapping functional groups.

Gene expression patterns associated to the pattern of *Salmonella* colonization in liver

A striking difference between the expression patterns during normal and disturbed development were the expression profiles itself, smooth (normal development, clusters 6-9) versus irregular (disturbed, clusters F-I) expression patterns. Clusters A to E have similar smooth expression profiles as observed in control chickens. The expression patterns in clusters G, H and I all exhibited an irregular decline in gene expression at 2 dpi. Whereas cluster F displayed an irregular peak in expression at 2 dpi. When plotting the average gene expression pattern of the clusters F, G, H, and I separately together with the *Salmonella* counts in liver or caecum, a remarkable similarity in timing between gene expression and *Salmonella* counts in the liver could be observed (data not shown). These data suggest a correlation (G, H and I) and anti-correlation (F) between these irregularities and the *Salmonella* count in the liver. Thus the passing of *Salmonella* over the intestinal barrier to colonize the liver, results in marked changes in gene expression patterns of epithelial cells. Expression of multiple genes involved in a variety of processes in the jejunum seem to be inhibited or delayed (cluster G-I) or accelerated (cluster F) due to the transmigration of *Salmonella*. Apparently, from 4 dpi onwards biological processes return into their 'normal' mode. This could be due to the fact that the jejunum becomes mature enough to sustain invading *Salmonella*, resulting in a more normal gene regulation and a decrease of bacterial counts in the liver.

Functional processes

Genes involved in functional processes were characterized by high expression immediately after hatch and decreasing expression over time [5]. The processes associated with functional development were (almost) similar between disturbed and normal development, as indicated by the high expression of metabolic and biosynthetic processes early in life (cluster 3 and C), high expression later in life (cluster 8 and H) and the constant expression level around the normalized zero value (cluster 5 and E). These clusters display metabolic processes, such as 'localization/transport', 'cell migration/motility', 'cytokine biosynthetic process' and 'negative regulation of biosynthetic processes'. Therefore we speculate that these processes are due to feed intake rather than a consequence of the *Salmonella* disturbance. This coincides with earlier observations in which immediately after hatch high activity of different digestive enzymes such as maltase, aminopeptidase, dipeptidase [126, 127] and disaccharidase [128] is measured. However some functional processes might also be delayed, caused by the presence of *Salmonella*. For example *Salmonella* has the ability to disturb the

normal production of cytokines by its immuno-modulation capacity [129]. In cluster H the groups 'cytokine biosynthetic process' and 'negative regulation of cytokine biosynthetic process' are observed, which were not observed in normal jejunal development. This suggests that the presence and systemic invasion of *Salmonella* alters the expression of some genes in these cluster. However most functional processes are still active in a similar fashion as observed in normal jejunal development.

Morphological processes

During normal jejunal development, genes involved in morphology were highly expressed immediately after hatch, whereas at day 21 of age normalized expression levels of the morphology related functions fluctuate around zero [5]. In contrast to normal development, during a *Salmonella*-disturbed development genes were low expressed directly after hatch which could reflect an underlying morphological process, whereas at the same time high expression of cell turn-over processes was observed. This indicates that the disturbance by *Salmonella* greatly affected the early morphological development of the chicken jejunum. The clusters A, B, D, G and I contain functional groups related to morphological processes.

Cell development and differentiation are important processes for the morphological development of the intestine. Thus the dominant presence of such processes might be because *Salmonella* disturbs jejunum integrity immediately after infection, which causes high turn-over of cells. This is consistent with the observation that *Salmonella* causes necrosis of cells present in the jejunum, especially of macrophages [130] and the assumption that a proper functioning jejunum requires a turn-over of jejunal cells. The expression of several of these 'morphological' gene groups seems to be associated to the pathology induced by *Salmonella*, for example the gene groups involved in cytoskeleton or adhesion related processes. Regulation of the cytoskeleton is most probably directly related to the *Salmonella* disturbance, since it is known that cellular invasion of *Salmonella* is associated with major changes in the cytoskeleton [131]. Apoptosis and proliferation on the other hand are processes needed for recovery and development of the jejunum.

Thus compared to control intestinal development morphological processes are different in the disturbed intestinal development, especially in terms of timing. Genes involved in developmental processes are low expressed early in time, which suggests that *Salmonella* delays the morphological jejunal development. Furthermore the immature intestine is not yet able to respond properly to a

Salmonella invasion [132] compared to the mature intestine, and therefore *Salmonella* is able to invade more easily.

Immunological processes

During normal development immunological processes were predominant and displayed well recognized and well-organized spatial-temporal expression patterns with low expression early in life and from 4 days post hatch a steadily increasing expression [5]. After *Salmonella* disturbance we expected to find predominant immunological processes. Therefore, it was very surprising to observe that in contrast to normal development, no clustering of immunological processes was found during the *Salmonella* disturbed development. Instead, expression of immune related genes was observed in clusters D, G, H and I. In all these clusters multiple genes are involved in 'Response', as well as genes involved in 'T-cell activation'.

When we focused on the various immunological KEGG pathways, the general expression pattern in these pathways is disturbed in the *Salmonella* infected chickens compared to control chickens. The well-ordered spatial-temporal pattern, of low to high gene expression, in immunological pathways of normal chickens is disturbed by *Salmonella*. Moreover no rapid and immediate high induction of genes is found in the chosen pathways, while the chickens did showed clear clinical signs of infection, based on colonization to liver and weight. As observed in normal developing chickens, immunological processes do occur in infected chickens, however these immunological processes are scattered over different clusters based on their gene function.

The early positive expression of $CD8^+$ cells suggests that they are needed earlier in the infected chickens. However at 8 dpi overall gene expression stays behind, in comparison to control chickens. Moreover in infected chickens at 21 dpi for both TCRαβ and TCRγδ a lower number of positive cells is observed, in comparison to control chickens. Thus in the end the disturbance by *Salmonella*, not only leads to a less smooth development of immune related genes also the at 21 dpi the number for $CD8^+$ cells, TCRαβ and TCRγδ cells is lower. We also observed significant differences in TCRαβ 2 and 21 dpi, which are not seen in the KEGG immunological pathway. This latter observation could be due to the fact that the TCRαβ and TCRγδ are not the only receptors for this pathway, therefore the overall general expression pattern could differ because of other input. These stimuli could for example be from ICOS and CD28 or PDCD1 and CTLA4, which are known co-stimulatory or inhibitory signals respectively.

Thus as observed in normal developing chickens, immunological processes do occur in infected chickens, however these immunological processes, as validated with immunohistochemistry are scattered over different clusters based on their gene function.

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4

Jejunal Gene Expression Patterns Correlate with Severity of Systemic Infection in Chicken

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Abstract

Background: Not much is known about the effect of *Salmonella enteritidis* on changes in the developmental processes occurring in the intestine of young chicken. Therefore we investigated the correlation of intestinal gene expression patterns with the severity of systemic *Salmonella* infections.

Methods: The number of *Salmonella* colony forming units (CFUs) in the liver of infected chicken were plotted against the average intestinal expression profiles of previously identified gene expression clusters. The functional properties of all the genes taken together present in 3 clusters exhibiting positive correlation at early time-points were compared with the functional properties of the genes displaying antagonistic correlations in 1 cluster. The top 5 ranking functional groups were analyzed in further detail.

Results: Three clusters showed gene expression profiles which were positively correlated with the severity of systemic disease as measured by the number of *Salmonella* colony forming units in the liver. In these clusters, genes involved in morphological processes were predominantly present. One cluster had a profile that was negatively correlated with the severity of systemic disease, as measured by numbers of CFUs in the liver. The genes in the latter cluster were mostly involved in cell turn-over and metabolism.

Conclusions: In the developing jejunum of young chicken, both stimulatory and inhibitory gene expression mechanisms are correlated with the severity of systemic *Salmonella* infections.

Key words: Jejunum, Severity of infection, *Salmonella*, gene expression, hatchlings

4.1 Background

Intestinal development is hallmarked by functional, morphological and immunological development. Genes involved in these three categories have different spatial temporal expression patterns, as observed in earlier studies [1-4]. Developmental studies are mostly performed in healthy chickens and not much is known of the effect of a disturbance on intestinal development. In a previous [5] study we disturbed intestinal development by oral infection with *Salmonella* and studied the effects of *Salmonella* on the immunological development of the intestine for 8 hours post infection (pi) until 21 days pi. Besides the immunological pathways we identified clusters of genes whose expression was correlated with the severity of systemic infection (numbers of CFUs in the liver). In this study, we further analyzed this correlation and focused on the functional properties of genes displaying the correlated expression profiles only in the earlier time points until 4 days pi. This study contrasts other studies that use *Salmonella* infected chicken to study (innate) immune responses or differences in susceptibilities [6-8].

4.2 Methods

Design

We used the dataset E-MEXP-2042 from ArrayExpress [9,10], describing whole genome transcriptional profiling of chicken jejunum in a time series (8 hours until 21 days pi) after orally infection with *Salmonella*. The data from Schokker *et al.* [5] was used to define average expression profiles of 9 clusters of genes and to identify the 4 clusters that showed expression profiles correlated with *Salmonella* counts in the liver. Here we investigate the differences between the positive and negative correlation to the trait in more depth in the first 4 days pi. We combined the genes of the clusters G, H and I (2,169 probes), which were found to possess a positive correlation early in time to severity of systemic disease and compared this group of genes to the genes present in cluster F (791) which showed an opposite correlation pattern to the severity of systemic disease. The Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 [11] was used for functional annotation clustering of these two sets of genes (March, 2010) [12]. The inputs were lists with chicken gene symbols or the homologous human symbols. Human has a better annotation, 29% of the probes mapped back to a human gene name compared to 24% to a chicken gene name. Also more databases are available for human and the data is analyzed with a human background. The settings in DAVID were similar to the settings as described by Schokker *et al.* [13].

4.3 Results and Discussion

The average gene expression profiles of the genes in clusters F, G, H, and I showed irregularities at 2 days pi compared to the smooth profiles of the corresponding clusters obtained from non-infected chicken. Clusters G, H, and I showed a positive correlation with the amount of *Salmonella* colony forming units (CFU) in liver, whereas cluster F showed an anti-correlation (Fig. 1). These positive and negative correlations are based on the period between 8 hours pi to 4 days pi. This correlation suggests that the irregularity in gene expression patterns is most probably directly related to the systemic invasion of *Salmonella* from the intestinal tract.

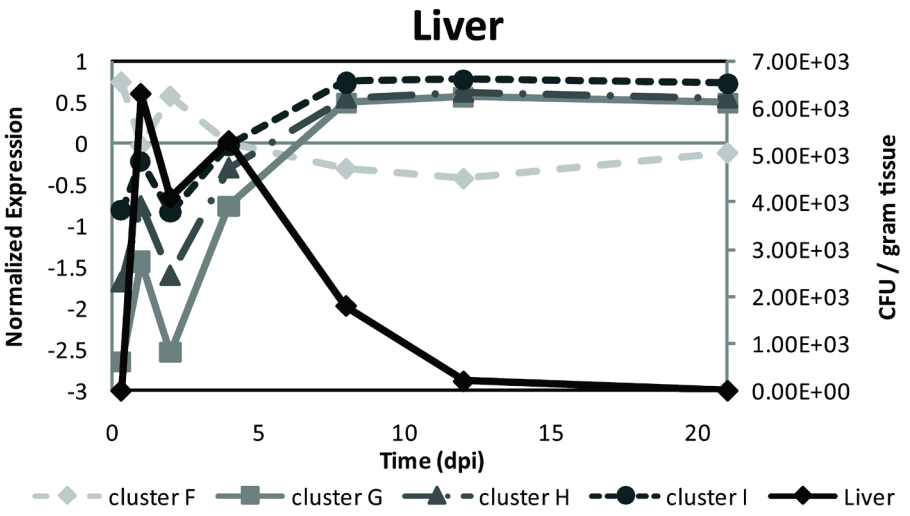

Figure 4.1 Correlation of average gene expression patterns of genes present in clusters F–I with *Salmonella* CFU per gram liver tissue in time

Cluster F shows negative correlation with the number of Colony Forming Units (CFUs) in liver from 0.33-4 days post infection (dpi), whereas clusters G, H and I have positive correlation in that time period.

The correlations further suggest that these associated genes encode for functional properties related to the severity of systemic disease. To investigate the functional properties, clusters G, H and I were grouped, because of their similar expression pattern and similar association to the number of CFUs in the liver. Subsequently, the genes residing in positively or negatively correlated clusters were used as input for the functional clustering analysis by DAVID. The resulting top 5 of the functional annotation clustering is depicted in Table 4.1.

Table 4.1 DAVID Functional Annotation Clustering Top 5

Correlation	Rank	Functional Group	ES	Count
Negative	1	Thrombospondins	2.564950833	6
	2	Cellular homeostasis	2.078669595	16
	3	Regulation of activity / Metabolic process	1.969787156	14
	4	Programmed cell death	1.894708614	20
	5	Ion homeostasis	1.825373132	10
Positive	1	Calmodulin (IQ domain)	3.840704976	12
	2	Fibronectin	3.688368085	18
	3	Contractile fiber	3.664863955	15
	4	Cell morphogenesis (neuron)	3.419394841	22
	5	Immunoglobulin I-set	3.397685801	17

Abbreviations: ES, Enrichment Scores

Most functional groups in the positively correlated expression profiles are related to morphological processes, like 'fibronectin', 'contractile fiber' and 'cell morphogenesis'. Another top 5 group is 'Calmodulin (IQdomain)', which is involved in multiple processes, like metabolism, inflammation and intracellular movement. Thus this positively correlated group is characterized by major processes involved in both morphological and immunological functions. Apparently, due to transmigration of *Salmonella* many genes involved in morphological related processes are regulated. Increased transmigration from the gut correlates with increased expression of a number of genes involved in morphological processes in the jejunum, whereas decreased transmigration of *Salmonella* leads to lower expression of such genes.

Also a specific immune related process 'immunoglobulin I-set' is present among the top 5 list. However, the genes contained in this domain are mainly involved in cell adhesion processes. For example vascular (VCAM), intercellular (ICAM), neural (NCAM) and mucosal addressin (MADCAM) cell adhesion molecules, as well as junction adhesion molecules (JAM) [14]. Some of these genes are also involved in immune cell adhesion, for example ICAM1 and VCAM1 are involved in monocyte-endothelial adhesion [15]. Moreover JAM genes are known to be involved in lymphocyte homing [16]. The expression of these adhesion genes is directly and

positively related to *Salmonella* transmigration and the severity of systemic disease.

In the negatively correlated group high expression is observed at 8 hours pi, followed by stable expression around zero until 21 days pi. Compared to the corresponding cluster of non-infected chicken, in the *Salmonella* disturbed chicken this cluster showed an irregularity in gene expression profile opposite to the *Salmonella* load in liver. This peak in gene expression may reflect a feedback mechanism of the jejunum. In this cluster, functional groups like 'thrombospondin', homeostasis related processes and 'programmed cell death' are observed, which are mainly involved in cell turn-over processes. The thrombospondin family is related to adhesive glycoproteins, and is involved in various processes like adhesion/migration, cytoskeletal organization, proliferation, phagocytosis, apoptosis and platelet aggregations [17,18]. The functional annotation clustering results furthermore indicate that in this cluster, genes are also involved in programmed cell death. After infection with *Salmonella* at day zero different processes are initiated and also the *Salmonella* load increases in liver (Fig. 1). Many cells will die or loose functionality due to the infection, therefore it necessary for the host to respond by replacing infected and affected cells. Thus the majority of genes residing in the negatively correlated group are associated to cell turn-over processes suggesting that due to the transmigration of *Salmonella* across the intestinal mucosa, processes for tissue repair are induced almost immediately. However the data suggest that the genes involved in such processes have a delayed response to the *Salmonella* infection, therefore a peak in gene expression is observed after the peak of *Salmonella* load in the liver.

In addition to the turn-over processes, also processes involved in 'homeostasis', maintenance of an internal steady-state at the level of the cell are observed. These latter processes can be characterized as metabolic.

4.4 Conclusions

Severity of systemic *Salmonella* infections can be associated to gene expression patterns in the jejunum. Negatively correlated gene expression patterns correspond to processes involved in metabolism, cell turn-over and tissue repair. Positively correlated gene expression patterns are associated with morphological and immunological related processes.

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5

Differences in the Early Response of Hatchlings of Different Chicken Breeding Lines to *Salmonella enterica* serovar Enteritidis Infection

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Abstract

Poultry products are the major source of food-borne *Salmonella* infection in humans. Broiler lines selected to be more resistant to *Salmonella* could reduce the transfer of *Salmonella* to humans. To investigate differences in the susceptibility of newly hatched chicks to oral infection with *Salmonella enterica* serovar Enteritidis, 3 commercial broiler lines (A, B, and C) were infected immediately after hatch and compared to healthy controls at 0.33, 1, and 2 d post infection. Weight, bacteriological examination, and the jejunal influx of CD4, CD8, TCRαβ, TCRγδ, and KUL01 (macrophages and dendritic cells) cells that are positive was investigated. In addition, the jejunal transcriptional response was analyzed using whole-genome chicken cDNA arrays. *Salmonella* colony-forming unit counts from cecal content and liver revealed that *Salmonella enterica* entered the body at 0.33 d post infection. Broiler line A appeared most susceptible to intestinal colonization and the systemic spread of *Salmonella*. In addition, the *Salmonella*-induced jejunal influx of macrophages in this line showed a clear increase in time, which is in contrast to lines B and C. On the other hand, all lines showed a peak of $CD4^+$ cells at 1 d post infection when infected chicks were compared to control chicks. The transcriptional response of line A clearly differed from the responses in lines B and C. Functional analysis indicated that the majority of the differentially expressed genes at 0.33 d post infection in line A were involved in cell-cycle functions, whereas at 2 d post infection the majority of the differentially expressed genes could be assigned to inflammatory disorder, differentiation and proliferation of (T) lymphocytes. These data indicate that hatchlings of different broiler lines differ in their systemic spread of *Salmonella* and suggest that intestinal barrier functions, as well as immunological responses, may be the underlying factors. We hypothesize that the differences between genetic chicken lines divergent in their response to *Salmonella* infection at a young age include developmental differences of the gut.

Key words: broiler line, *Salmonella enterica*, susceptibility, early response

5.1 Introduction

Salmonella enterica is one of the most wide spread serovars causing foodborne infections [1, 2]. Consumption of Salmonella contaminated poultry derived products is a major source for salmonellosis in humans [1]. Broiler lines selected to be more resistant to *Salmonella*, could reduce its transfer to humans. In chicken, *S. enterica* causes both systemic disease as well as symptomless infections [3]. Especially chicks at young age develop systemic infections. An initial infection of the small intestinal lining by *Salmonella* can expand rapidly from the gut to internal organs [4]. Chicken salmonellosis is characterized by diarrhea and dehydration, growth depression, and a high mortality rate [5].

Although salmonellosis causes substantial economic losses to farmers, poultry breeders have so far mainly focused on production traits, rather than the susceptibility to infectious diseases. With the recent restrictions and discussion on the wide-spread use of antibiotics in animal husbandry, resistance to *Salmonella* in hatchlings has a high priority. As a side-effect, commercial broiler lines have developed variable susceptibilities to *Salmonella* [6-8]. Genetic resistance to salmonellosis is a polygenic phenomenon, involving multiple genes (reviewed by Wigley [9]). Genetic association studies with *Salmonella* are restricted by the number of available potential markers [10-12]. Nevertheless genetic markers associated to *Salmonella* resistance have been identified, such as Toll-like Receptor 4 (TLR4), Solute Carrier 11A1 (SLC11A1), Interleukin 2 (IL2), inducible Nitric Oxide Synthase (INOS), Caspase1 (CASP1), Cluster of Differentiation 28 (CD28), Inhibitor of Apoptosis Protein 1 (IAP1) and prosaposin (PSAP) [13]. Since the chicken genome has been sequenced [14], the genomic approach has opened new opportunity to study chick biological processes in more detail, including the host response to *Salmonella* infection and the search for resistance markers.

The objective of this study was to investigate differences in the susceptibility of newly hatched chicks of three different commercial broiler lines to the systemic spread of *Salmonella enterica* serovar Enteritidis after oral infection and to identify the potential underlying mechanisms.

5.2 Material and Methods

Chicken

140 one-day-old male broiler type chickens per different commercial breed (line A, B, and C) were obtained from a commercial breeder (Pronk's Boerderij, Meppel, The Netherlands). At day 0 of age the chickens were randomly divided in two groups of 70 animals each. These were housed in separate ground cages under a

strict 12-h light cycle and chicken had access to a standard commercial chow diet and water ad libitum. All chicken experiments were approved by the institutional animal experiment committee, in accordance with the Dutch regulations on animal experiments.

Infection and dissection

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) phage type 4 (nalidixic acid resistant) was grown in buffered peptone water (BPW) overnight at 37°C [15]. Immediately after hatch (0 days post infection (dpi), chicken were checked to be free of *S.* Enteritidis by bacteriologic examination of cloacae swabs. Seventy chickens were infected with *S.* Enteritidis at hatch by oral inoculation (0.2 ml bacterial suspension, diluted in saline) containing 10^5 CFU (colony forming units) from an overnight culture. The remaining 70 animals received 0.2 ml saline (control group). At 0.33, 1, 2, 4, 8, 12, and 21 dpi, 10 chicks (per infection state and per broiler line) were sacrificed by cervical dislocation. Body weights were measured (n=10) and jejunal sections were snap-frozen in liquid nitrogen and stored at -70°C for immunohistochemistry (n=10) and RNA isolation (n=6). Aliquots of caecum luminal content (n=10) were harvested for CFU determination (serial dilution plating on brilliant green agar + 100 ppm nalidixine plates). Liver was removed (n=10), weighed and kept at 4°C, until bacteriological examination on the same day as sectioning, by plating. These samples were also collected for the following time-points 0.33, 1, 2, 4, 8, 12 and 21 dpi. The average of 10 chickens was calculated for each time-point.

Bacteriological examination

For *S.* Enteritidis detection in intestine and liver, one gram liver or caecal content was homogenized in 9 ml PBS, serial diluted and plated on Brilliant Green Agar with 100ppm nalidixic acid (BGA-Nal+). After 24h aerobic incubation at 37°C, log-transformed CFU counts were determined. These samples were collected for 0.33, 1, 2, 4, 8, 12 and 21 dpi. The average of 10 chickens was calculated for each time-point.

Immunohistochemistry

Eight µm thick jejunal cryosections, collected at 0.33, 1, 2, 4, 8, 12, and 21 dpi, were stained with specific antibodies using an indirect immunoperoxidase staining method as described by Schokker *et al*. [16]. In brief, slides were treated for endogenous peroxidase activity, blocked with BSA and incubated for with monoclonal antibodies against $CD4^+$ cells, $CD8^+$ cells, TCRαβ, TCRγδ or macrophages

(CT-4;1:200, CT-8;1:200, TCR2;1:50, TCR1;1:400 and KUL01;1:50, respectively, Southern Biotech, Birmingham, Alabama), followed by peroxidase-conjugated rabbit anti-mouse Ig (P0161, Dako, Denmark). Peroxidase activity was detected by 3,3-diaminobenzidine and sections were counter-stained with haematoxylin. Negative controls were performed by omission of primary antibody. Three consecutive representative microscopic images per section were captured and analyzed using Image-Pro Plus (version 6.2, Media Cybernetics, Bethesda, MD, USA). Positive stained cells were counted, averaged per time point and group, and represented as positive cells per tissue area and percentage increase compared to the respective control.

RNA isolation

Total RNA was isolate using TRIzol reagent (Invitrogen, Breda, The Netherlands), according to manufacturer's instructions. In brief, 50-100 mg frozen jejunal samples were homogenized in liquid nitrogen, using a mortar and pestle, and suspended in 1 ml of TRIzol by passing 10 times a 21-G needle. After centrifugation (12,000×g for 10 min at 4°C), RNA was extracted with chloroform, precipitated in isopropanol, was washed and dissolved in diethylpyrocarbonate-treated water. RNA quality and integrity was checked by spectrophotometer (ND-1000, Thermo Fisher Scientific) and accepted when A260/A280 ratio>1.8, and when 5S, 18S and 28S ribosomal bands were visible after agarose-gel electrophoresis without signs of degradation.

Microarray hybridization

ARK-genomics Gallus gallus 20K v2 single spotted 20,460 oligonucleotides (corresponding to 24182 different chicken genes/transcripts; ARK Genomics, Roslin, Midlothian, UK) were used for differential expression analysis. To create homogeneous groups, RNA of 6 jejunal samples obtained at 0.33, 1 and 2 dpi of chickens infected with *S.* Enteritidis with similar weights and similar liver CFU counts were selected and labeled. Each chick RNA (n=6) was co-hybridized with RNA of a common RNA reference pool with equal amounts of jejunal RNA from 54 control and 54 infected chicken obtained at 0.33, 1, and 2 days of each broiler line. In total 54 slides were hybridized, 6 infected chicken x 3 lines x 3 time-points. Total RNA (5 µg) was labeled and hybridized using the MICROMAX TSA labeling and detection kit (PerkinElmer, Wellesley, MA, USA) according to suppliers instruction with minor modifications as described by Van Hemert *et al*. [17]. On each slide RNA of an infected chicken (Cy3-labeled) was hybridized with a sample of the reference pool (Cy5-labeld) or vice versa (dye-swab). The choice of a common reference pool

in the experimental design results in the identification of transcriptional differences between the lines rather than individual chicks. Furthermore the use of a two color array approach requires hybridization signal on a spot to calculate the fold change. This experimental design minimizes the number of empty spots on the array. After signal amplification, the microarrays were dried and scanned for Cy5 and Cy3 fluorescence intensities using an Axon GenePix® Microarray Scanner and GenePix Pro 6.1 software (Molecular Devices, Sunnyvale, CA, USA).

Analysis of microarray data

Detected spots were corrected for local background and normalized per slide using GenePix software. On the 38 slides passing our visual quality check, RG intensity/density-plots and MA-plots check, inter slide variations were normalized using Print-tip LOESS and quantile normalization in R-package Limma (Linear model for microarrays; http://www.r-project.org). Genes per group at time point 0.33, 1 and 2 dpi were compared to the reference pool (n=5 microarrays for line A; 1 and 2 dpi, and n=4 microarrays for the remaining groups) and log Fold Changes and p-values were calculated in R-package Limma (multiple testing across contrasts using empirical moderated Student's t-test and F-test statistics) and represented as log2 (Cy5/Cy3) intensity ratios. Genes were considered statistically differential expressed when the false discovery rate (FDR) adjustment of the p-values was less than 0.1 with a cut off of |logFC|>1. Likewise differential gene expression patterns between different broiler chicken lines per time point were studied. For each comparison, 4 or 5 values were obtained per gene, with 1 to 3 dye-swaps per group. Microarray data are available in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE27069.

Functional and annotation (cluster) analysis on data were performed, using DAVID [18], Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA), GeneCards® (www.genecards.org), and UniProtKB (www.uniprot.org). As human genes are functionally better annotated compared to chicken (approximately one third of the chicken probes map back to gene identifiers), human orthologous were used in for the IPA analysis. This resulted in 14,580 mapped out of 20,458 Entrez Gene identifiers (EGIDs).

Statistics

The data are expressed as mean±SEM. Comparisons were regarded significant when $p<0.05$. Unless stated otherwise, p-values were assessed using two tailed Student's t-test.

5.3 Results

Body weight, and bacteriological examination

In the first 2 dpi, *S.* Enteritidis infected chicks of all lines showed weight depression compared to their age matched controls (Fig. 5.1 and supplementary table 1 (see online version)). During the next 19 days, infected lines kept gaining weight, although in most cases growth ratios and body weights lagged behind compared to control animals. Percent wise, weight gain of infected line A was lower compared to the healthy chicks of line A, varying from 88% to 101%. In infected line B a similar, slightly higher percent wise weight gain pattern was observed (89%-101%), whereas the percent wise weight gain of line C varied from 108% to 94%.

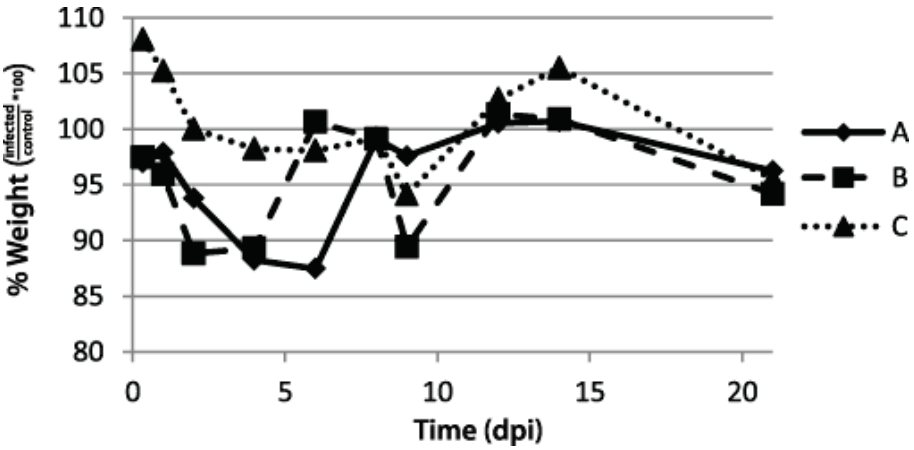

Figure 5.1 Body weight development over time for 3 commercial broiler lines (A, B, and C) infected with *Salmonella enterica* serovar Enteritidis
The BW are plotted versus time as days post infection (dpi). The BW is displayed as the percentage of weight (weight of infected chicks divided by that of control chicks times 100). Each value represents the mean of 10 sampled chickens.

Although replication of *S.* Enteritidis was apparent from caecal content plating's at all time-points (Fig. 5.2, lower panel), infected chicks were free of clinical symptoms. Chicks residing in the control group were free of *S.* Enteritidis throughout the experiment (data not shown). The results of CFU counting's after liver suspension plating's confirmed the transmigration of *S.* Enteritidis from the gut to the liver in all three chicken lines, which was indicative for a systemic infection (Fig. 2, upper panel). At 1 dpi liver CFUs counts were significantly higher for line A compared to line B ($p<0.03$) and line C ($p<0.001$). Also at days 2 and 4 the number of liver CFU's was much higher in line A compared to lines B and C. Only at

8 dpi line A reached values comparable to those of lines B and C. Line A reached a maximum of approximately 8,000 CFU per ml 2 dpi, whereas line B peaked at 4 dpi (2,000 CFU/ml), and line C at 8 dpi (3,000 CFU/ml). The caecal bacteriological load did not show any significant differences between lines, and therefore will not be discussed further.

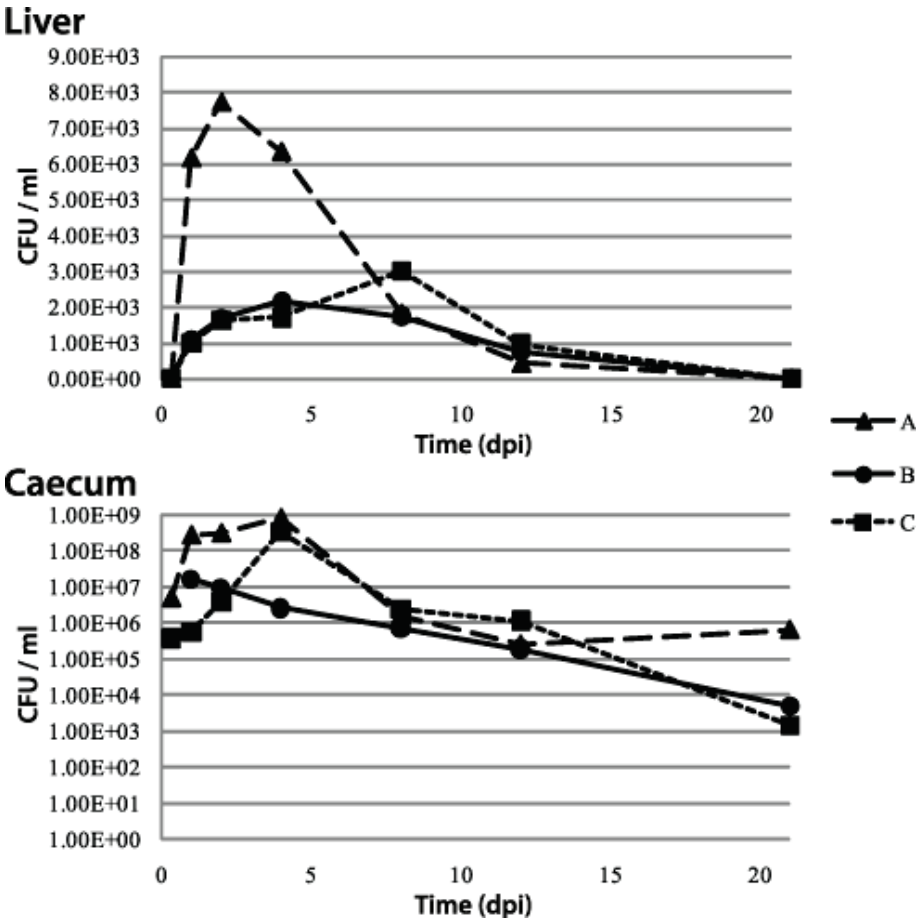

Figure 5.2 The number of *Salmonella enterica* serovar Enteritidis colony-forming units over time in the liver and ceca of 3 lines of broilers (A, B, and C)
The number of colony-forming units per milliliter of tissue (y-axis) versus time as days post infection (x-axis) is plotted for both. Each value represents the mean of 10 sampled chickens.

Immunohistochemistry

To examine differences in the number and influx of immunological cells in the jejunum of infected animals between the three lines, KUL01$^+$, CD4$^+$, CD8$^+$, TCRαβ$^+$ and TCRγδ$^+$ cells were visualized by immunohistochemistry. The results are shown as percentage of their line specific controls in Figure 5.3. A higher increase of KUL01$^+$ cells at 2 dpi was observed in line A (200%) compared to lines B and C (both 120%). In all three lines the influx of CD4+ cells peaked at 1 dpi (line A and B: 350%; line C: 250%). In the first dpi differences in CD8$^+$ cells counts compared to the control were minimal. However 2 dpi line C showed a decrease, whereas line A and B showed an increase of CD8$^+$ cells of respectively 175% and 250%. For TCRαβ$^+$ cells, all the three lines displayed a different pattern over time when comparing infected with control chicks. In line A infected chicks had less TCRαβ+ cells at 0.33 and 2 dpi, and a maximum of 140% at 1 dpi. The pattern of line B was the opposite of line A, where at 1 dpi a minimum of 75% was observed, followed by an increase of 200% 2 dpi. Infected chicks from line C had an increase of TCRαβ$^+$ cells over time, resulting in a 130% more cells compared to control chicks at 2 dpi. The TCRγδ$^+$ cells from line A showed a similar pattern as the TCRαβ$^+$ cells. However the numbers of TCRγδ$^+$ cells in line A at 0.33 dpi were lower compared to line B. Infected chicks of line B had approximately 150% more jejunal TCRγδ$^+$ cells compared to their controls at 0.33 and 1 dpi, but less TCRγδ$^+$ cells were observed at 2 dpi. The TCRγδ$^+$ cells from line C showed a similar increasing pattern as observed for TCRαβ$^+$ cells, when comparing infected with control chicks, which resulted in a 200% increase of TCRγδ$^+$ cells at 2 dpi.

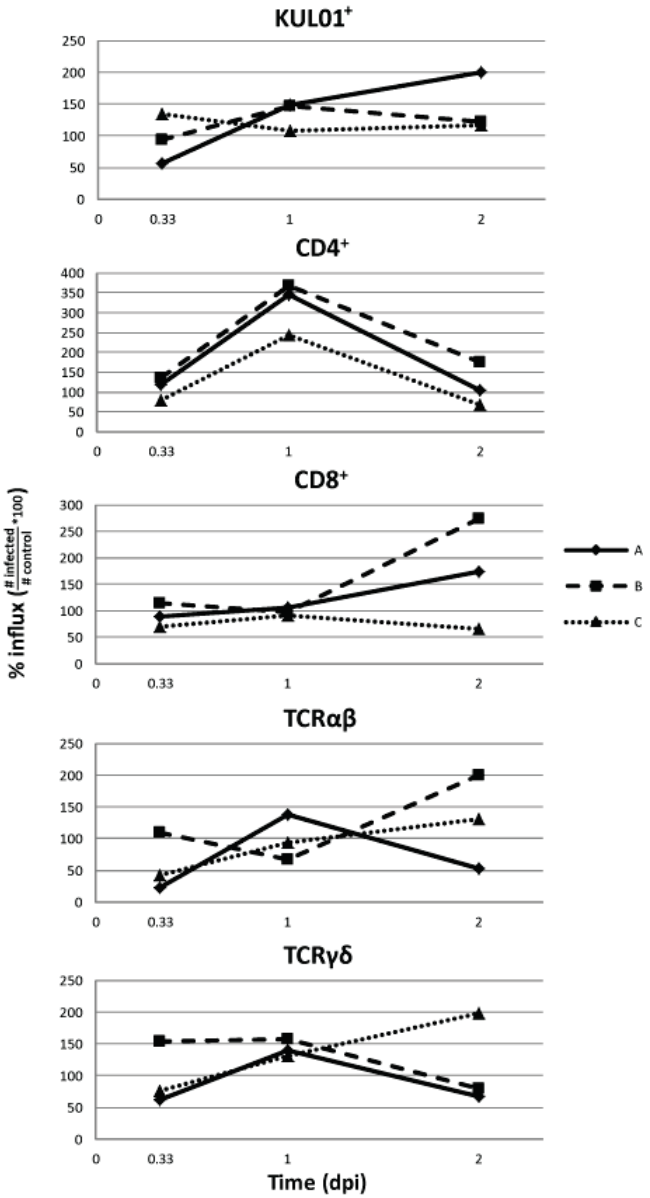

Figure 5.3 Staining of different immune cells in the jejunum of broilers infected with *Salmonella enterica* serovar Enteritidis at 0.33, 1, and 2 d post infection

The percentage of influx (number of infected cells divided by the number of control cells times 100) in the intestine is depicted on the y-axis, and on the x-axis is the time as days post infection (dpi). These measurements were performed for all 3 broiler lines (A, B, and C). Each value represents the mean of 10 sampled chickens.

Intestinal transcriptional profile

The transcriptome of infected lines A, B and C were compared to a common RNA-reference pool, to get insight into the differences and similarities of transcriptional responses to *S.* Enteritidis. The application of common reference RNA pool provides an internal control and normalizes differences in hybridization parameters and array variations. This experimental design enables the direct comparison between the chicken lines at 3 time-points, and is less useful to examine the reaction patterns of a single chicken line upon an infection with *S.* Enteritidis.

Changes in the jejunal gene expression were evaluated and the number of differential expressed genes are shown in Table 5.1 (FDR<0.1). Transcriptome analysis of line A at 0.33, 1 and 2 dpi identified 68, 1 and 77 differential expressed genes, for line B 2, 0 and 3 differential expressed genes, and for line C 4, 0 and 0 differential expressed genes. This indicates that the transcriptional response in line A is quite different from the responses in lines B and C. Three genes were significantly differently (FDR<0.1) expressed in all lines, RIGG01844 (LOC415756), RIGG04848 (LOC422305) and, RIGG13934 (HBAA), which may be indicative for a partial common response between the lines. RIGG01844 was found in line A at 0.33 dpi and in line B at 2 dpi, RIGG04848 in line A at 0.33 dpi and in line B at 0.33 dpi, and lastly RIGG13934 in line A at 0.33 dpi and in line C at 0.33 dpi. Although these probes have been mapped to the given names, no specific functions have been assigned to these genes yet.

For line A, the most significant up and down regulated genes could be separated into distinct patterns of gene expression over time. The genes induced at 0.33 dpi were not found to be induced at 1 dpi. However, at 2 dpi a completely different set of genes was induced in this chicken line. Due to the limited number of differential expressed genes, functional cluster analysis only revealed results for 0.33 and 2 dpi. At 0.33 dpi 25 out of the 46 down regulated genes had a logFC< -2 whereas only 6 up-regulated genes had a logFC>2. Using DAVID functional cluster annotation, the top three clusters were identified at both 0.33 and 2 dpi (Table 5.2), namely ‘cell cycle phase/nucleotide binding’, ‘transferase/phosphate metabolic process’ and protein localization/transport’ at 0.33 dpi, and ‘regulation of synaptic and impulse transmission’, ‘T cell activation’ and ‘guanyl-nucleotide exchange factor activity’ at 2 dpi. Because the human genome is much better annotated compared to the chicken genome, we used the human orthologues of the avian gene identifiers for the functional and biological interpretation of our data. Although this is not an ideal situation, it is currently the most promising method for functional annotation in chicken [19-21].

Table 5.1 Number of differentially expressed genes (false discovery rate (FDR) adjustment of P-value < 0.1) among 3 broiler lines (A, B, and C) infected with *Salmonella enterica* serovar Enteritidis

Broiler	Time (dpi[a])	FDR<0.1 # genes		
		up	down	total
A	0.33	22	46	68
	1	0	1	1
	2	45	32	77
B	0.33	0	2	2
	1	0	0	0
	2	1	2	3
C	0.33	0	4	4
	1	0	0	0
	2	0	0	0

[a] dpi, days post infection

The IPA database mapped 43 humanized gene IDs, whereof 30 were assigned to one or more functions or diseases (Table 5.2). At 0.33 dpi 'cell cycle/division' and 'cancer processes' were significant, whereas at 2 dpi the following processes or disorders were significant 'neurological disorder', 'nervous system development and function', 'inflammatory disorder', 'cell morphology / shape change', and 'digestive system disorder'. IPA networks suggest a key regulatory role for 17β-estradiol, retinoic acid, dihydrotestosterone and vascular endothelial growth factor (VEGF).

Table 5.2 Functional clustering
Clustering by DAVID and a selection of the most prominent significant enriched functions in IPA (p<0.01 and # genes≥4), for both 0.33 and 2 days post infection (dpi) in line A.

Analysis	dpi[1]	ES*	Function	Genes
DAVID[2]	0.33	1.03	Cell cycle phase/nucleotide binding	13
	0.33	0.63	Transferase/phosphate metabolic process	10
	0.33	0.61	Protein localization/transport	5
	2	1.14	Regulation of synaptic and impulse transmission	6
	2	0.88	T cell activation	7
	2	0.85	Guanyl-nucleotide exchange factor activity	3
IPA[3]	0.33	7.98E-05	Cancer processes	18
	0.33	1.15E-02	Cell cycle / cell stage	7
	2	1.61E-04	Nervous system development and function (growth of axons)	4
	2	2.15E-04	Inflammatory disorder	22
	2	2.54E-04	Digestive system disorder	14
	2	7.40E-03	Cell morphology / shape change	6
	2	9.82E-03	Neurological disorder	22

[1]dpi = days post infection.
[2]DAVID (Dennis *et al*., 2003).
[3]IPA = Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA); selection of processes with the P-value < 0.01 and the number of genes being at least 4.

5.4 Discussion

In this study we provide evidence that hatchlings of three different broiler lines differ in the systemic spread of *S.* Enteritidis after infection via the oral route. Line A appeared to be more affected by *S.* Enteritidis than lines B and C. Compared to lines B and C, the transcriptional responses in line A at 0.33 and 2 dpi were associated with cell cycle and immunological processes, suggesting that aspects of these processes are the main differences between lines A and B/C in their response to *S.* Enteritidis.

The cell cycle arrest found in line A with gene expression analyses probably depresses the intestinal development, epithelial renewal and enterocyte activity in line A, resulting in impairment of the intestinal barrier. Apparently, the presence of *S.* Enteritidis in the gut of line A results in a gene expression pattern that inhibits

the normal development of the gut and that weakens the intestinal barrier. This results in an enhanced transmigration of *S.* Enteritidis to other organs as well as creates an environment in the lumen for higher replication of *S.* Enteritidis or easier colonization or both. Line A is also different in its response to *S.* Enteritidis from lines B and C with regard to some aspects of its immunological response. Among the 22 differential expressed 'inflammatory' genes at 2 dpi in line A, 11 are also assigned to digestive and endocrine disorders and several other genes to neural and connective tissue development. Some known key players in the immune response were found to be up regulated in line A, at least in a different way compared to lines B and C, including CD7, CD80, ICOS, MARCO, MASP2 and RIPK2. After bacterial recognition, MARCO can activate the complement system through MASP2 [22, 23] leading to CD80 expression on monocytes and B cells, and of ICOS which is involved in co-stimulation of T cells. ICOS enhances all T cell responses to foreign antigens, from cell proliferation, dendritic phagocytosis, secretion of lymphokines, to mediation of cell-cell interaction [24]. Moreover down regulation of RIPK2 and CD7 inhibits the innate and adaptive immune system, NF-κB activation, apoptosis [24, 25] and T cell (B cell) interactions during early lymphoid development [26]. Apparently, the immunological response of the intestinal tissue of line A to a *S.* Enteritidis infection is quite different from that in lines B and C and is heavily reprogrammed due to the infection. In our approach genome-wide expression was investigated in *S.* Enteritidis infected chicken at early age, Kogut and colleagues already showed the importance of several immune related genes [27] and Toll-Like Receptors (TLRs) [28].

In contrast to line A, lines B and C both have a lower systemic *S.* Enteritidis spread, a strong influx of $CD4^+$ cells, and increasing numbers of $TCR\alpha\beta^+$ cells. Furthermore, transcriptome analysis did not provide evidence for a significant deviation of the transcriptional response of lines B and C from the transcriptional profile of the reference pool RNA. This indicates that there are no major differences in the transcriptional responses of the respective lines to the other lines. But a few transcriptional differences of lines B and C are observed, namely the genes CD72 and Rgc32 for line B, and HBB, HBAA, HBE for line C. The transcriptomic data of line B accounts for some limited immunological responses that are different from the other chicken lines. The observed up regulation of CD72, 2 dpi may account for enhanced B cell activation and B-T cell interaction, but also decreased IFN-γ release, reducing macrophages activity [29]. The observed down regulation of Rgc32 (2 dpi), stimulates the mitotic G2/M (G2 phase (third and final sub phase of interphase) / M (mitotic) phase) transition and reduces inflammatory responses. Furthermore, a strong influx of $CD4^+$ cells and a down-regulation of the

inflammatory response, based on the expression of Rgc32, were observed for this line. Microarray data of line C revealed that three hemoglobin subunits were down regulated at 0.33 dpi, and steadily increased over time, to higher levels at 2 dpi. A similar, although non-significant increase, was observed in line A, which indicates for a common regulatory pathway. In line B these hemoglobin subunits are already expressed at a high basal level at 0.33 dpi. Furthermore hemoglobin subunits can also function as antioxidants [30]. In part the hemoglobin expression pattern is in accordance with the number of KUL01^{+} cells macrophage counts, where the number of macrophages increases at 2 dpi compared to the control situation.

In this study we detected the transmigration of *S.* Enteritidis from the gut to the liver in hatchlings of three commercial chicken lines. This is in agreement with our current knowledge on the morphological and functional development of the gastro-intestinal tract of chicken. Usually, the epithelial lining together with the intestinal innate and adaptive immune system form a sufficient defense line to prevent systemic infection. Another study already showed that different lines react differently to a *S.* Enteritidis infection. By crossing commercial birds with different non-selected chicken lines, enhancement of innate immunity could be established [31]. At hatch, however, intestinal morphological (mass, villi number, length, enterocyte number, crypt depth and proliferating cells) and functional changes occur [32]. It takes at least 4 days before jejunal crypt and villi are matured, making the chicks of young age much more susceptible to trans-epithelium migration of pathogens.

The data described in this paper are in agreement with a previous study in which it was shown that differentially expressed genes between two chicken lines may be involved in determining the resistance to *S.* Enteritidis [33]. The transcriptional and immunological data presented here, suggest that aspects of intestinal integrity, as well as immunological responses may be the underlying processes for the enhanced systemic spread of *S.* Enteritidis at hatch in line A. However, it cannot be excluded that line specific differences result in a higher jejunal *S.* Enteritidis CFU, which, may contribute to differences in the efficiency of trans-epithelial migration of pathogens. The observations described in this paper indicate that broilers from different lines respond differently to *S.* Enteritidis infection. Lines B and C appear to be more resistant at hatch compared to line A. Especially the bacteriological and transcriptomic data, suggest that this is most probably due to a more mature intestine in lines B and C at the time of infection.

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6

Shift in Chicken Intestinal Gene Association Networks after Infection with *Salmonella*

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Abstract

A primary infection of *Salmonella enteritidis* causes a spatial-temporal dependent change in the gene expression patterns in the intestine of chickens (*Gallus gallus*). This is the result of a dynamic intestinal response to adapt to the altered environment and to optimize its 'health' and functionality under the new circumstances. By inferring gene association networks (GANs), the complexities of and changes in biological networks can be uncovered. Within such GANs highly interacting (hub) genes can be identified, which are supposed to be high-level regulators connected to multiple processes. By exploring the intestinal expression of genes differing between control and *Salmonella* infected chicken in a time-dependent manner differences in GANs were found. In control chickens more developmental processes were observed, whereas in infected chickens relatively more processes were associated to 'defense/pathogen response'. Moreover the conserved protein domains of the identified hub genes in controls were nuclear-associated, whereas hub genes in infected chickens were involved in 'cellular communication'. The shift in topology and functionality of the intestinal GANs in control and *Salmonella* infected animals and the identification of GAN-specific hubs is a first step to understand the complexity of biological networks and processes regulating intestinal health and functionality under normal and disturbed conditions.

Key words: Hubs, Jejunum, *S.* Enteritidis, Chicken, Gene Associated Network

6.1 Introduction

Salmonella enterica serovar Enteritidis [1] is a pathogen, which causes an infection especially in young chickens. After ingestion, *Salmonella* colonizes the intestine and invades the intestinal mucosa [2] which subsequently induces an array of innate and adaptive immune responses. Infection with *Salmonella* Enteritidis rarely causes mortality in chickens that are more than 1 month old [2]. However, in young chickens this infection leads to diarrhea and an influx of heterophils (equivalent of mammalian neutrophils) together with intestinal inflammation and damaged epithelial cells [3]. The mobilized heterophils are known to play an important role in restricting *Salmonella* to the gut and protection against the development of systemic disease [4]. By the application of gene expression studies, many genes are already known to be involved in the chicken response after challenge with *Salmonella*. A number of these studies have contributed to the current understanding of the effect of *Salmonella* on the host response [5-9]. These genome-wide functional studies indicated that a number of genes, signaling pathways and effector mechanisms, associated with different immunological processes and cells, including T cells, are activated by the infection process in order to combat the invading pathogen and restore tissue homeostasis. In addition, *Salmonella* infections in young chicks also affect normal development and metabolic functioning of the intestine by changing the expression of a huge variety of genes and processes [10]. It is likely that all these immunological and non-immunological pathways and processes are connected in time to each other in global gene association networks and that they are orchestrated by a limited number of high-level 'regulators', the so called hub genes. Thus genes that are regulated at time point X, influence gene induction at time point X+1. However, such time-dependent, global gene association networks have not been generated so far for complex tissues and high-level tissue 'regulators' have not been identified, although they have the potential to orchestrate an array of genes or processes. In a dynamic and heterogeneous multi-cellular tissue, such as a developing intestine, this type of regulators are expected to include global transcription factors and molecules involved in intra- and inter-cellular signaling and communication. Therefore, identification of candidate high-level regulators might be of importance for the development of strategies to modulate intestinal homeostasis.

A major drawback of many gene expression studies is that they only provide 'snapshot views' of the gene association networks controlling the (transcriptional) response of biological systems. Also for *Salmonella* infected chicken, time-

dependent regulation of gene expression has poorly been investigated and has been a major limitation in the modeling of gene association networks. Such networks are critical to begin to understand the behavior of a developing chicken intestine under normal and severely disturbed conditions. Therefore the objective of this study was to make an effort to generate gene association networks describing the transcriptional response of chicken intestinal tissue in time under two highly contrasting conditions and to identify and characterize candidate high-level regulators.

6.2 Material and Methods

The study was approved by the 'Dier Experimenten Commissie' (DEC), in accordance with the Dutch regulations on animal experiments. The registration number is 2006044.b and the experimental number 2006048.

Animal experiment

Broiler male chickens were orally inoculated at day of hatch with 0.2 ml phosphate buffered saline (PBS) containing 1×10^5 *Salmonella* enteritidis (nalidixic acid resistant strain of *Salmonella enterica* serotype Enteritidis PT4 isolated from chicken [1] or with PBS (control group). At seven consecutive time-points 8 hours post infection; 1; 2; 4; 8; 12; and 21 days post infection, jejunal whole tissue samples, liver and ceaca were taken of 5 chickens of the *Salmonella* infected group and 5 chickens of the control group, all samples for RNA analyses were immediately snap frozen in liquid nitrogen. Bacteriological colonization assays were performed for liver and caecum, which showed a peak of approximately 8×10^8 colony forming units (CFUs) at 4 days post infection (dpi) for caecum and at 2 dpi approximately 6,000 CFUs for liver. No CFUs were found at any time point in the control group (data not shown, see Figure 1 in Schokker *et al.*, 2010). RNA was isolated from whole intestinal tissue samples as described before. Intestine from individual chickens intestine was homogenized in TRIzol (Invitrogen, Breda, The Netherlands) and RNA was isolated. This RNA was purified using the Macherey-Nagel NucleoSpin® RNA II kit (April 2007/Rev. 07).

Microarray data

The intestinal RNA used for microarray analyses consisted of seven time-points: 8 hours post infection; 1; 2; 4; 8; 12; and 21 days post infection of *Salmonella* and control chickens. At each time-point 10 chickens were sacrificed, 5 control and 5 *Salmonella* infected chickens, resulting in a total of 70 samples that were independently used for microarray analyses. RNA was labeled following Agilent

protocols and was individually, in a single dye design hybridized to Agilent 4×44K chicken arrays (AMADID 015068). The Agilent protocols (GE1-v5_95_Feb07) were strictly followed for all procedures, including hybridization, washing, staining and scanning [11, 12]. All hybridizations were performed in a controlled environment and one batch of dye was used to decrease the variation between arrays. Moreover the data for both control and *Salmonella* infected is MIAME compliant and the raw data is available at ArrayExpress, accession number E-MEXP-2042. All 70 slides were loaded and quantile normalized simultaneously. The normalized data is depicted in tab-delimited Supplementary File 'Norm_Exp_Combined.7z'.

N-way ANOVA

Within GeneSpring GX (v.10.0.2 build 85765), an N-Way ANalysis Of VAriance (ANOVA) was performed to determine the concurrent effect of N parameters. In this particular case two parameters and their interaction term were determined, thus we performed a 2-way ANOVA. This assesses the individual influence of each parameter as well as their net interactive effect, both calculated by type-III sum of squares (SS) [13, 14]. The type-III SS is defined as follows: Let T and G be the factors, containing different levels. The complete model for these factors is yijk = μ + ti + gj + cij + eijk, where yijk is the k-th observation in ij-th treatment group, μ the grand mean, ti(gj) the additive combination and cij and eijk the error term, which accounts for the variation in y that cannot be explained by the other terms on the right hand side of the equation. The difference in residual sum of squares (RSS) of the models, yijk = μ + ti + gj + cij + eijk and yijk = μ + gj + cij + eijk is the SS corresponding to factor T. Besides factor T, this difference in RSS between the full model and the model excluding that factor, can be performed for factor G and C (interaction term). In our analysis, a 2-way ANOVA was performed between 'time' (0.33, 1, 2, 4, 8, 12 and 21 days post infection (dpi)), 'group' (control or infected) and 'interaction time-group'. Within GeneSpring the option 'pairs of conditions' was used, furthermore we selected the following pairings; 'control 0.33 dpi' vs. 'infected 0.33 dpi', 'control 1 dpi' vs. 'infected 1 dpi', etcetera. Thus probes which are significant under a certain threshold differ between control and infected on a certain time-point. All probes were used as input and an asymptotic p-value computation with a Benjamini-Hochberg multiple testing correction was performed. Results are summarized and depicted in a Venn-diagram (Figure 6.2). Probes which were significant under a p-value of 10^-5 (759 probes) in the time-group interaction were selected as input for the network. Such a stringent cut-off, p<10^-5, was used to decrease the number of probes which were used as input for the GeneNet software, because Opgen-Rhein and colleagues showed that the

linearity assumption of the VAR model is satisfied when using approximately 800 nodes as input [15].

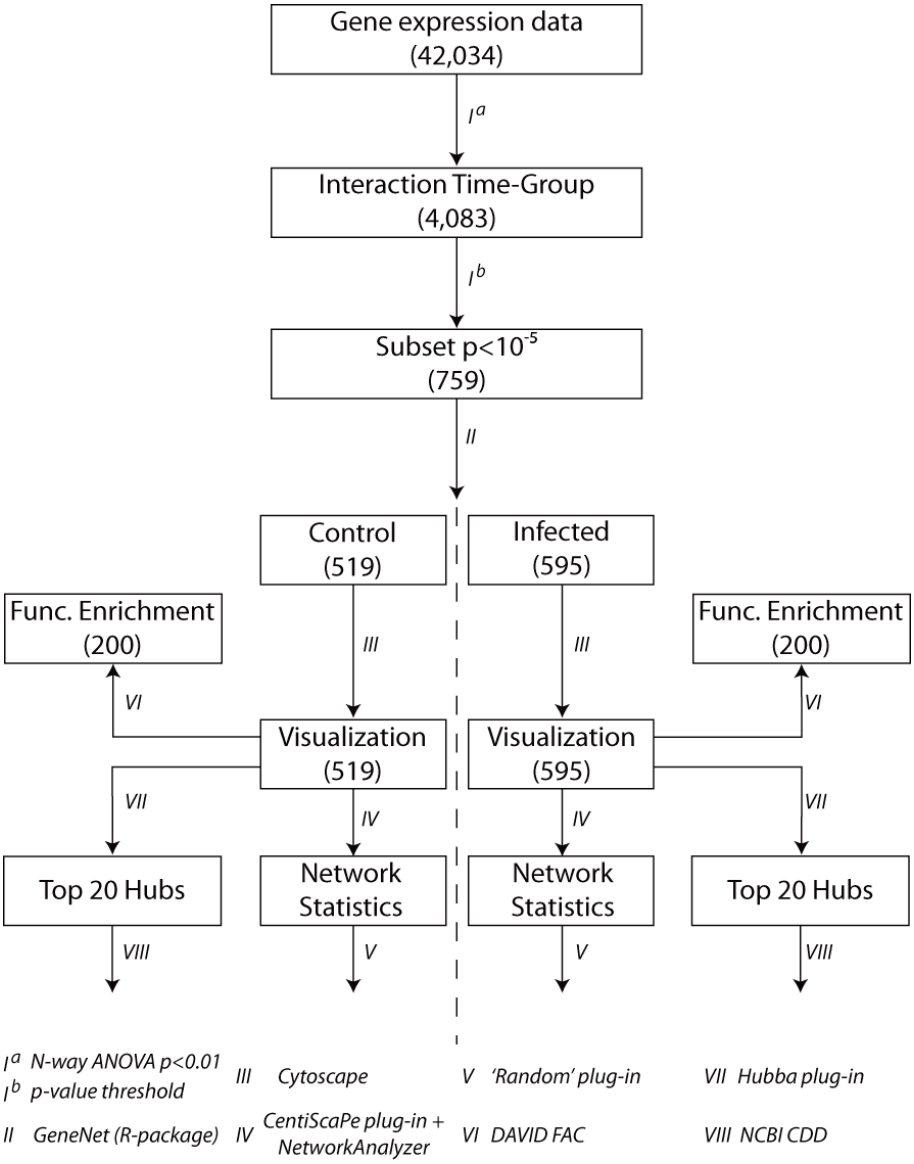

Figure 6.1 Pipeline of network generation and analyses

Figure 6.1 (continued) Pipeline of network generation and analyses
Different filtering steps and analyses are depicted. Eight different consecutive analyses were performed: Ia) N-way ANOVA on all probes and Ib) a more stringent p-value cut-off to limit the number of probes considered; II) the specific probe subset was used in 'GeneNet' for control and *Salmonella* infected chickens separately; III) resulting GANs were visualized with Cytoscape; IV) various network statistics were calculated by different methods; V) random scale-free networks were generated; VI) for both GANs residing genes were functionally annotated and clustered; VII) for both networks the top 20 ranking hubs were calculated; and VIII) the protein sequences of the identified hubs were investigated for the presence of known protein domains. The number of nodes/genes which is present at that stage is indicated between brackets. The various R tools and Cytoscape plug-ins which were used at the different steps are indicated by roman numbers.
Abbreviations: Func., Functional; DAVID FAC, Database for Annotation, Visualization and Integrated Discovery: Functional Annotation Clustering; NCBI CDD, National Center for Biotechnology Information: Conserved protein Domain Database

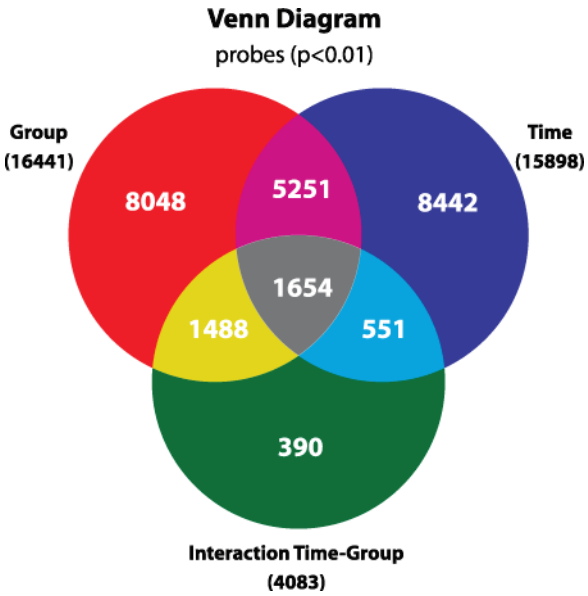

Figure 6.2 Venn diagram of 2-way ANOVA categories
Probes which are under the a p-value threshold of 0.01 are divided over three categories; 'group' (red), 'time' (blue) and 'interaction time-group' (green).

Gene networks

Gene networks were derived using the R-package GeneNet (v.1.2.3), which is publicly available on the Comprehensive R Archive network (CRAN) website (http://cran.r-project.org/) as well as on Strimmer's laboratory website (http://strimmerlab.org/software/genenet/). This package was used to derive high-dimensional dependency networks from genomic data [15-18]. The consecutive algorithm steps are: 1) determine correlation matrix by use of shrinkage estimator (and define whether the data is longitudinal); 2) calculate partial variances and correlations; 3) infer partial correlation graph; 4) conduct multiple testing (FDR); and 5) construct directed network. In the current version of GeneNet, putative directions to edges in the network are also assigned. The data was loaded and set as longitudinal, with 5 repeats for every time point. Furthermore, in the control data the correlation shrinkage intensity, lambda, was estimated at 0.4478 and the variance shrinkage intensity, lambda_var, at 0.4103. In the *Salmonella* infected situation the lamba was estimated at 0.0885 and the lambda_var at 0.0514. All these analyses were performed in the R-environment, version 2.9.0. The resulting networks were visualized by Cytoscape (v.2.6.2) [19], the top 1000 edges for both situations were depicted, resulting in 519 nodes in control and 595 nodes in infected chickens. Note that because of the poor annotation of the chicken genome, multiple probes representing the same gene were not combined. Thus it is possible that in subsequent analysis a gene can be represent more than once.

Further analyses using Cytoscape plug-ins

CentiScape 1.1

This tool was used to calculate different network centrality parameters, which were used to compare the data-driven networks. CentiScape [20] also provides a Boolean logic based tool, as well as characterization of nodes whose topological relevance depends on more than one centrality. Furthermore this plug-in is completely integrated into Cytoscape and can be freely downloaded at http://chianti.ucsd.edu/cyto_web/plugins/ index.php. Before analyzing the networks separately, the isolated nodes in the control network were removed. In the infected network no isolated nodes were observed.

Random network

Scale-free networks are most commonly observed in biology [21, 22], and these networks are compared to our chicken gene expression networks. The aim is to investigate whether 'real' data has similar aspects and components as observed in scale-free random networks. The following simulation settings were used: the

number of nodes was set to 519 for control or 595 for infected, minimum edges per node were 2 and initial number of nodes was 3.This plug-in can be downloaded at http://groups.google.com/group/cytoscape-announce/browse_thread/thread/31b7d20b062f6f3b?pli=1 and has three main components, but we only used 'generating random networks according to three different models'. Because our focus is on generating random scale-free networks, as described by Barabasi and Albert [23], we only used component 1.

Hub calculations

We used the package HUBBA within the Cytoscape environment, where for each node different topology-based scoring methods were calculated. The hubs identified in the networks are based on their 'bottleneck' score, which is used for exploring the importance of nodes in interaction networks by different topology based approaches. In this study probes with the top 20 highest 'bottleneck' scores are defined as hubs and were ranked by this score from high score (rank 1) to low score (rank 20). The Bottleneck Score is calculated as described by Pržulj *et al.* [24], where for each node v in the interaction network, a tree of shortest paths is constructed originating form node v. The Hub Objects Analyzer (HUBBA) package [25] was used for exploring important nodes in an interactome network. This method is based on graph theory and publicly available at http://hub.iis.sinica.edu.tw/Hubba.

Basic Local Alignment Search Tool (BLAST)

A mapping and annotation step was performed for the hub genes, because information for these probes was lacking. This was based on the location of the probe sequence in the chicken genome (NCBI Build 2.1) after performing BLAST [26-28]. The following steps were performed: 1) all 40 sequences (38 unique) from the hub genes were modified to a FASTA-format, 2) blastn from NCBI BLAST was used [26-28], 3) the unique sequences were queried with the following options in the database: refseq_genomic, organism: Gallus gallus (taxid: 9031) and optimize for highly similar sequences (megablast), 4) the results were parsed using BioPerl [29]. The resulting genome locations were visualized in NCBI's Gallus gallus (chicken) genome Map Viewer (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9031). This is summarized in Supplementary Table S1 (see online version).

Functional annotation clustering

The Database for Annotation, Visualization and Integrated Discovery (DAVID) [30] was used for functional annotation clustering. Within DAVID (2008) different data sources are combined and analyzed by heuristic fuzzy multiple-linkage partitioning [31]. For the genes present in each network a separate functional annotation clustering was performed. The input was a list of chicken genes symbols which was converted to its homologous human symbol. Because human has a better annotation and more databases are available, all the clusters were analyzed by choosing a human background. For every functional grouping an enrichment score was calculated, by the following formula:

$ES = -Log(\sqrt[n]{p_1 * p_2 * .....p_n})$, where ES is the enrichment score, and p_1, p_2 and p_n are the individual p-values of the associated database term.

Stringency was set to high, which implies that similarity term overlap was set to 3 and the corresponding threshold to 0.85. Furthermore the initial group membership, as well as the final group membership was set to 3, and the multiple linkage threshold was set to 0.50. The default data sources and their corresponding databases were used.

Conserved Domain Database (CDD)

To characterize the hub genes of both control and infected chickens, we investigated conserved domains in their protein sequence using the Conserved Domain Database [32, 33].

6.3 Results

Gene selection and inferred networks

By performing the 2-way ANOVA method we identified which probes were significant when testing for difference in 'group' (control versus infected), 'time' (8hpi – 21dpi) or the 'interaction time-group'. When a probe is significantly different when testing for control versus *Salmonella* infected, the probe will be present in 'group'. Significant for 'time' means that a probe differs in one or more time-points compared to the whole time-series. If the net interaction effect of 'time' and 'group' is significant, this means that these probes can have different effects (Figure S1). Each factor ('group' and 'time') is assessed individually, as well as the net interactive effect of the two factors ('interaction time-group'). All probes were evaluated for 'group', 'time' and 'interaction time-group', resulting in 16,441 probes for 'group', 15,898 probes for 'time', and 4,083 probes for 'interaction time-

group', with all probes being significant under a corrected p-value of 0.01 (Figure 2).

In total 4,083 probes obtained of the input of *Salmonella* and control data of all time points reside in the 'interaction time-group' and this genes have the potential to regulate each other because these genes showed interaction with time and infection status and are therefore the most promising genes for building association networks. From this we generated a sub-selection of 759 probes ($p<10^{-5}$), because we decided to focus only on genes which display a contrast between control and *Salmonella* infected chickens in time with a high significance. These 759 probes were used to generate the networks for both control and *Salmonella* infected chicken and used as input for GeneNet [15-18]. Only the top 1,000 most significant edges, with a corresponding FDR<0.1 (Figure S2) were visualized with Cytoscape [19]. In the control situation 519 nodes were present in the GAN out of the 759 probes used as input, whereas 595 nodes were present in the GAN of the *Salmonella* infected situation. Because we only visualize the top 1,000 edges, not all nodes are represented in the networks, the remaining 240 (control GAN) or 164 (infected GAN) nodes are not connected to the 519 (control GAN) or 595 (infected GAN) nodes with this threshold. A total of 406 nodes were present in both visualized networks. Several nodes were only observed in one GAN, meaning that such probes interact highly depending on the context of the circumstances, presence or absence of *Salmonella*.

To confirm whether these networks represent and resemble biological networks, the inferred GANs were compared with 'random' scale-free networks. By inferring 'random' networks, we can validate whether our data driven networks resemble scale-free network aspects and topology. The random networks consist of 519 or 595 probes, similar to the control and *Salmonella* infected GAN. The 'real' networks and 'random' constructed networks were compared based on their network statistics (Suppl. Table S2 (see online version)). Moreover a loglog-plot was made for the node degree distribution, to check visually whether 'real' networks were scale-free (Figure S3). The graphs show that the 'real' data have similar patterns to scale-free networks, which follow a power law. When we compared 'random' versus 'real data' GANs differences were observed, especially when taking the in- and out-degree of nodes into account. The hubs and central nodes of the 'real' networks have more outward than inwards edges, which is expected since it indicates that when the gene expression of a central node or hub changes, all the connected genes will also change expression [34]. This phenomenon was not observed in the random scale-free GANs, where hubs and central nodes contained more inward edges than outward. Thus generating random networks helps in

identifying the topological structure of scale-free networks, but in random networks the directionality of edges is (biologically) less relevant. Because nodes containing many inward edges are likely to be involved in complex interactions of this node, for example genes associated to metabolism, like amino acid biochemistry [34]. Although the directionality is putative, in our networks complex interactions are subordinate to regulatory interactions, because mostly outward edges are observed.

A comparison between control and infected GANs was made to investigate differences between various network parameters (Suppl. Table S2 (see online version)). Most parameters are in a similar range, except for 'neighborhood connectivity' for which in the *Salmonella* infected network a higher connectivity is was observed. This indicates a higher level of cohesiveness in the network of *Salmonella* infected chickens compared to the control network. In biological terms this suggests that the gene association network of the *Salmonella* infected situation contains hubs which have more or stronger interactions with other genes compared to the control situation.

Functional annotation

To further validate the biological relevance of the GANs and to identify differences in gene composition and functionality between both GANs, a DAVID Functional Annotation Clustering was performed. Here this analysis also serves as validation of the relevance of the established networks. The control network consists of 519 nodes (probes) of which 200 map back to a gene symbol. However only a subset of 154 identifiers was recognized by DAVID. Similarly, in the *Salmonella* infected network 595 probes were present, which map back to 216 gene symbols and DAVID recognized a subset of 167 identifiers (Table 6.1).

Table 6.1 DAVID Functional Annotation Clustering top 10

GAN	Functional Group	ES	Genes involved
CONTROL	Localization / Transport	3.23	43
	Regulation of cellular component organization and biogenesis	1.63	3
	Cell migration / Motility	1.45	8
	Calcium transport	1.45	4
	Cellular localization (establishment)	1.44	14
	Transporter activity	1.43	19
	In folding membrane / Vesicle formation	1.38	8
	Metabolic / Biosynthetic process	1.37	3
	Channel activity / Transport	1.35	10
	Regulation of variety of activities (protein kinase / catalytic)	1.24	8
INFECTED	Localization / Transport	1.47	37
	Fibronectin	1.4	5
	Biological regulation	1.27	52
	Receptor / Signal transducer activity	1.24	30
	Adhesion / Integrin	1.24	4
	Regulation translation and biosynthetic process	1.15	5
	Transmembrane region	1.13	35
	Response (immune and wounding)	1.09	9
	Helix-loop-helix DNA-binding	*0.94*[a]	4
	Tetratricopeptide region	*0.93*[a]	4

[a] In *Italic* are terms with an enrichment score below one and are thus not significant. Abbreviations used are: GAN, Gene Association Network; ES, Enrichment Score.

In both control and *Salmonella* infected chickens the functional group 'Localization / Transport' has the highest Enrichment Score. In the control network relatively more genes were involved in metabolic processes, for example the genes represented in the metabolic related functional groups: 'Metabolic / Biosynthetic process'; 'Regulation of cellular component organization and biogenesis'; and 'Regulation of variety of activities (protein kinase / catalytic)'. Also genes involved in regulatory processes like 'Transporter activity' were observed. The control network also encompassed a number of developmentally related functional groups such as 'Cell migration / Motility' and 'Cellular localization (establishment)'. In the *Salmonella* infected network a significant portion of the genes was also found to be

involved in metabolic related process like 'Regulation translation and biosynthetic process', as well as regulatory processes, like 'Biological regulation' and 'Receptor / Signal transducer activity'. However, in contrast to genes residing in the control network, relatively more genes in the *Salmonella* infected network were involved in cell mobilization and defense mechanisms as indicated by the presence of the following functional groups 'Fibronectin', 'Adhesion / Integrin' and 'Response (immune and wounding)'. Although a high overlap of genes is present in these lists of genes present in the visualized networks, differences are observed in functional annotation clustering groups.

Hub discovery and characteristics

For both GANs, genes that are highly connected to other genes, the so-called hub genes, were identified. Furthermore, a hub network was generated by visualizing the rank of each hub (Figure S4). To define whether these hubs encode protein domains associated with regulatory functions, like transcription factors, the hub genes were analyzed by the Conserved Domain Database (CDD) tool at NCBI. The hub network and the putative functions of hubs, based on the conserved protein domains, are depicted in Figure 6.3. A more detailed description of the domains and domain functions is given in Table S3 (see online version).

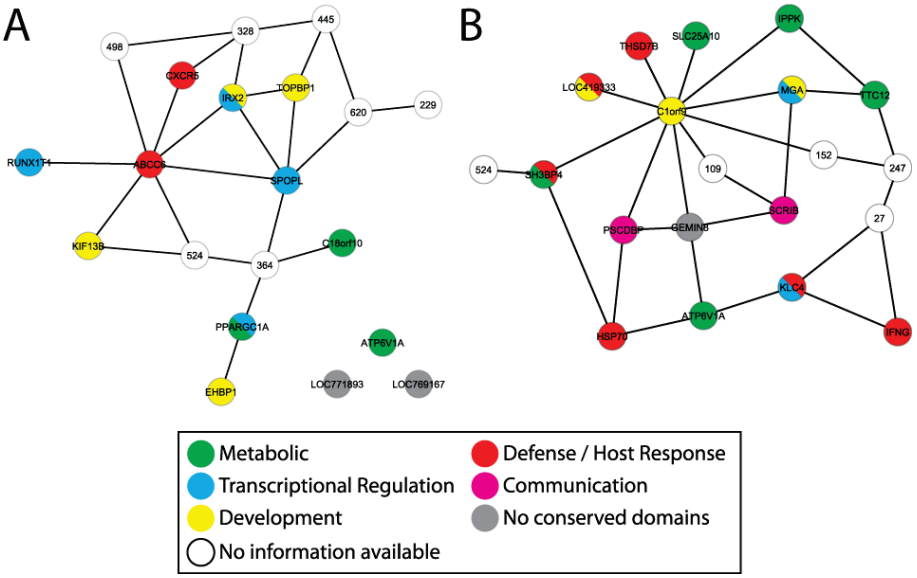

Figure 6.3 Network of top-20 'bottleneck' hubs, which are colored by their putative function

The top 20 'bottleneck' hubs and their (putative) function are depicted for control (A) and *Salmonella* infected chickens (B). The putative functions of the hubs are categorized in different group: Metabolic (green); Transcriptional regulation (blue); Development (yellow); Defense / Host response (red); Communication (magenta); No conserved domains (grey). For the white hubs no information is available at probe and/or domain level.

As indicated in Table S3 (see online version), the control GAN contains four hub genes with conserved domains which are associated to transcriptional regulation, like the MATH_SPOP domain [35], TAFH domain [36, 37], Homeodomain [38] and RRM domain [39].

In addition, four hub domains of the control network may be linked to development, Homeodomain [40], calponin homology domain [41], kinesin motor domain [42] and the BRCT domain [43]. The latter suggests that hubs containing these domains, regulate cell proliferation and differentiation processes. Two hub domains of the control network were related to metabolic functions, such as V_A-ATPase_A [44] and SMI1_KNR4 [45]. Finally, two hub domains of the control GAN were associated to other processes: the ABCC_MRP_domain2 [46] and 7tm_1 domain [47]. The ABCC_MRP_domain2 may participate directly in active transportation of drugs into sub cellular organelles or indirectly influence drug distribution [48]. The 7tm_1, 7 transmembrane receptor (rhodopsin family) domain is involved in signal transduction in response to hormones and neurotransmitters [49] and desensitization of receptors [50]. However, this domain resides within the

protein coding gene CXCR5, which is known to be involved in B cell migration and localization [51].
The GAN of the *Salmonella* infected intestinal tissue contains only one hub gene (MGA) with a conserved domain (TBOX) associated with transcriptional regulation. TBOX plays a critical role in development and has transcriptional regulatory activity [52]. In the *Salmonella* infected GAN relatively more conserved domain of the hubs were involved with defense and/or host protection, the IFNG [53], THSD1 [54], PTZ00009 [55] and Rab5 binding domain [56]. Also three conserved protein domains in 3 different hubs were identified which are possibly involved in development, such as SUN [57], FH2 [58] and TBOX domain [52].
Furthermore metabolic related conserved protein domains were also present among the hub genes of the *Salmonella* infected GAN, like V_A-ATPase_A [44], TPR [59], IPPK [60], mito_carr (SLC25A10) [61] and Rab5 binding domain [56]. Some hubs observed in this GAN may have a dual function, like SH3BP4 which might be involved in metabolic processes and/or 'pathogen response / host defense'. Because SH3BP4 is involved in internalization at the plasma membrane through a cargo-specific control of clathrin-mediated endocytosis, it may be involved in internalizing feed as well as bacterial components [62]. Another example is the PDZ_signaling domain, which is observed twice in the infected GAN, although in different genes, has a protein-protein recognition module and is involved in organizing polar sites of cell-cell communication [63].
Another observation was that in the control network, in comparison to the *Salmonella* infected network, hubs contain domains for proteins which regulate processes in the nucleus, thus focusing the system on intracellular communication. However in the *Salmonella* infected network, hubs contained protein domains which were predominantly involved in cytoplasmic and intercellular communication.

6.4 Discussion

Compared to common post-genomics analyses, like gene set enrichment analyses (GSEA) and Gene Ontology (GO) analyses, the approach of inferring gene association networks (GAN) from genomics data has the advantage that it can take longitudinal data into account and that the data can be analyzed at the systems level in time. In general, GANs provide a global overview of different but connected processes, but not of the underlying mechanism. To our knowledge GANs have thus been generated to get insight in the functional behavior of cells, but thus far not for getting insight in the behavior of complex tissues. In previous studies we explored

different intestinal gene expression patterns in time for either control or infected chickens [11, 12]. In the current study, a subset of genes which were significant in the interaction of time and group (absence or presence *Salmonella*) were investigated, by inferring a gene network based on co-expression and interaction of the genes and their proteins in time with other genes. The established GANs of 'healthy' and *Salmonella* infected chicken jejunum displayed a clear difference in topology, hubs and functionality. In contrast to GSEA, GAN analysis takes unknown probes into account and several of these 'unknown' genes were identified as hub. Therefore GAN analyses may help to (better) annotate (unknown) genes, like performing guilt-by-association and guilt-by-profiling analyses as described in yeast [64].

Networks and hubs

The generated GANs depict gene interactions based on linear gene co-expressions in time as determined with RNA isolated from whole jejunum containing a heterogeneous pool of cells. As said, up to now, most GANs have been generated from homogeneous cell population [65]. Here, we attempted to generate informative GANs for tissues that are highly dynamic and complex in terms of cell differentiation, cell function and cellular composition, and explored whether these GANs represent systems behavior. For that purpose we used two highly contrasting environmental conditions: a normal developing intestine and a developing intestine disturbed by a *Salmonella* infection. To focus on the major differences between the systems, stringent cut-off values were taken for the selection of genes that served as input for the GANs. This choice was also made because direct validation of the GANs representing complex systems is not possible for the moment. Our results demonstrate that through GAN analysis, it is indeed possible to obtain a first global view of highly regulated processes and the connectivity between them. We have several reasons that strengthen us to believe that the GANs represent, at least a part of, the biological behavior of the intestinal system. 1) The results of the functional analysis of GANs, are in agreement with earlier knowledge on development and effect of *Salmonella* infection from previous studies [7, 8, 11, 12, 66-68]. 2) The proposed functional properties of hubs are in line with expectations, in which hubs are involved in processes like transcriptional regulation or intra- and extracellular communication. 3) The topology of the established networks represents the topology of random scale-free networks with network centrality parameters which are expected for networks representing real biological phenomena.

We realize that our results require additional validation of the wiring and functions of the proposed association networks and the role of the proposed hubs in controlling the major system responses using independent methods. In principle, validation may be performed by promoter binding and correlated gene expression studies. However, such approaches are limited in their ability to validate models of transcriptional networks. A complementary strategy is to systematically perturb the expression of all the identified key regulator genes and monitoring the resulting cellular transcriptional responses, especially the change in the expression profile of the associated genes. This strategy has been successfully used in a cell-based mammalian system using mouse primary dendritic cells [65]. In such cellular systems gene perturbations can be performed by the use of large-scale RNAi perturbation experiments. In multicellular systems validation of transcriptional models depends on the availability of large collections of gene deletion mutants [69] and studying their effect on the expression of associated target genes. Unfortunately, in this case such validations are currently impossible to perform, because of the complexity of the used biological system and the lack of specific knock-out and knock-down mutants for chicken. The results of future studies focusing on genotype-phenotype relationships at the level of gene expression (eQTL) in different breeding lines may, however, be used for validation of the results presented here.

Network characteristics

At the time of these analyses, different methods were available to infer GANs, for example Bayesian and graphical Gaussian models (GGM). However in Bayesian networks it is impossible to incorporate cyclic regulations, like feedback loops [70]. These feedback loops have important biological functions, like ensuring homeostasis (negative feedback loops) or multistationarity (positive feedback loops) [71]. Approaches like dynamic Bayesian networks (DBNs) and GGM do not have these limitations [18] and the latter approach is very useful for the screening of causal relationships [70]. We used the GeneNet package [15-18] for inferring the data driven networks, because time-course data can be explicitly declared, although other methods do exist, like 'LASSO' [72] and 'minet' [73].

The network topologies of the control and the infected networks were quite different, suggesting a well-orchestrated system response under both conditions. Both networks displayed the characteristic features of biologically relevant networks, due to their differences when compared to the random scale-free networks. Both GANs showed a complex connectivity between genes. Our results suggest that major (high level) signaling events in complex tissues do not cascade

only via known biochemical pathways, but also occur via other unknown (indirect) routes that we do not understand yet. Apparently hubs play an important role in signaling; subtle alterations in expression of hubs may result in functional changes of the system.

Functional annotation clustering

Functional clustering of the nodes present in both GANs indicated different functionality and system behavior, generally in agreement with current knowledge. Because of the poor annotation of the chicken genome, we used human orthologues in the functional analyses. Nevertheless the functional clustering data give insight in global processes which differ between control and infected chickens. The control GAN is primarily dominated by genes and processes related to metabolism and development. This is plausible because in the developing small intestine digestion and absorption of food takes place, as well as maturation [74]. The developmental processes are absent from the high ranking functional groups of the network in *Salmonella* infected chickens. Instead, the *Salmonella* infected GAN is dominated by genes and processes involved in metabolic and 'defense / pathogen' responses. This observation is supported by the results of previous studies [7, 8, 11, 12, 66-68]. Furthermore the *Salmonella* GAN clearly shows the activity of a number of metabolic related processes, for example 'Localization / transport'. Apparently, digestion and absorption of food continues to take place, in both control and infected animals, although the genes involved displayed different expression patterns between control and *Salmonella* infected conditions. The functional groups 'Fibronectin', 'Adhesion and/or integrin' and 'Response (immune and wounding)' were found to be typical for the *Salmonella* infected GAN, indicating that the behavior of the systems in a '*Salmonella* environment' has a major focus on the invading pathogen. Due to these observations we believe that the networks represent relevant biological behavior.

Hub discovery and characteristics

Hubs are known to determine the behavior of the system [22]. By identifying hubs and characterizing their conserved protein domains (Suppl. Table S3 (see online version)), we obtained some more insight in the functions of the hubs. Strikingly, although a similar probe set was used for generating the networks, only two genes are represented in both top 20 hub lists: ATP6V1A and '524' (probe identifier: A_87_P030550). This explains the shift in topology between the control GAN and the infected GAN and is in line with a number of other transcriptional studies [7, 8, 66-68, 75, 76], where a comparison is made between control and *Salmonella*

infected chickens, all showing that perturbation with *Salmonella* have clear and significant effects. However, in the latter studies, the observed differences were based on observations on a fixed moment in time, whereas the GeneNet package takes into account that the data is temporal. Apparently, genes that function as major hubs in the control situation and that drive the behavior of the developing intestine are replaced or dominated by other hubs, in the *Salmonella* infected state. These hubs drive the intestinal system towards an adaptation to the new environment to optimize its 'health and functionality'. Many of the hubs in the control situation are transcriptional regulators, like transcription factors. Transcription factors are important regulators of the flow of transcription within host cells, an example are the Hox genes which are involved in specifying the body plan and regulating the host development [38]. In the top 20 of hubs in control chickens, the IRX2 gene was present, which contains a homeodomain and is likely to be involved in developmental processes. Furthermore the gene C18orf10 is involved in cell-wall formation and TOPBP1 which is associated to cell cycle checkpoint, indicate that the hubs found in the control situation are involved with developmental processes.

Several hubs residing in the *Salmonella* infected GAN have functions which are potentially involved in communication and cell signaling molecules. This suggests that in the control situation the major emphasis of the system is on the regulation of intracellular processes, whereas in the *Salmonella* infected situation nuclear regulation is reduced and replaced by cytoplasmic as well as extracellular regulation mechanisms. This suggests that on the systems level a shift from cellular activity and differentiation towards tissue remodeling and cell mobilization occurs. Although these results are not surprising by themselves, it demonstrates the value of the use of GAN analysis for whole tissue gene expression data. The possibility for identification of hubs or high level regulators, as shown in this study, may be of help in the selection of potential targets to modulate intestinal health and development, but also for modulation, prevention and/or treatment of intestinal perturbations. This requires the targeting of selected hub genes either by feeding, vaccination, and/or breeding strategies, which as indicated by the present study, may not be limited to immune-related genes.

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7

A Mathematical Model Representing Cellular Immune Development and Response to *Salmonella* in the Chicken Intestine

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Ready for submission

Abstract

The intestinal immune system of chicken consists of many different cells. The aim of this study was to create a dynamic mathematical model of the development of the cellular branch of the intestinal immune system during the first 42 days of life and of its responsiveness towards an infection with *Salmonella enterica* serovar Enteritidis. The system elements identified were intra- and extracellular *S.* Enteritidis bacteria, macrophages, $CD4^+$, and $CD8^+$ cells. The dynamics of twelve model elements were described by ordinary differential equations, including 50 parameters. During model development the parameter values were estimated from literature or from immunohistochemistry data. The model describes the immune development in none-infected birds well (average R-square of 0.87). The model shows less accuracy in predicting the immune response towards the *S.* Enteritidis infection (average R-square of 0.51), although model behavior was similar to the observed trends in time. However, with model evaluation against data from several independent experimental infections, strong deviations were observed. However with this developed mathematical model we show that model simulations allow the study of the effect of varying input parameters, as number of immune cells at hatch, on intestinal system outputs. Although the model was calibrated on a single chicken line, it was possible to simulate the behavior of other genetically different chicken lines. Model simulations have shown that this mathematical model has the potential to describe the effect of different input variables.

Key words: Mathematical model, Development, Immune, *Salmonella* infection, Chicken Intestine

7.1 Introduction

Intestinal health is prerequisite for sustainable poultry production. In this respect, it is important that the gut-associated immune system of young animals develops timely and appropriately. This development depends on a variety of genetic, nutritional, environmental, and management factors, which are usually investigated independently. No methods are available yet for studying the impact of these factors on immune development simultaneously. The availability of a mathematical model representing the major aspects of immunological development and responsiveness of chicken intestinal tissue may be a step forward in this respect.

After hatch the intestine develops rapidly and morphological, functional and immunological changes occur rapidly in time [1-3]. Development of the Gut Associated Lymphoid Tissue (GALT) is an important event, because chicken lack lymph nodes which are a rich source of immune cells (macrophages) in other species. The avian intestinal immune systems harbors a variety of immune cells including macrophages and dendritic cells [4, 5], plasma and (memory-) B cells [6], and an array of different T cell subpopulations, like helper T cells (T_H cells), cytotoxic T lymphocytes (CTLs), regulatory T cells (TREG cells), natural killer T cells (NKT cells) and memory T cells [7]. In addition, intraepithelial lymphocytes (IELs), which monitor the luminal content of the gut and eliminate distressed epithelial cells [8], are abundantly dispersed in the mucosal layer. Furthermore, lamina propria lymphocytes (LPL) are present, which are classified as differentiated effector lymphocytes and have a phenotype of activated memory T cells [9-11].

Specific components of microorganisms residing in the gut are monitored by Pathogen Recognition Receptors of the intestinal immune system, and when harmful microorganisms are detected, the innate part of the immune system is activated [12]. The process of immune activation in poultry has been the subject of numerous investigations. In these studies, members of the gram-negative *Salmonella* species are frequently used as the immune inducing agent [13-17]. These studies identified and characterized a variety of immunological components involved in response and defense mechanisms and defined the specific roles of immunological cells, signaling molecules, effector molecules, (signaling) pathways, and the products of a number of other genes [18-20].

It appears that the functionality of intestinal immunity is dependent on numerous components and complex interaction between these components and with the pathogen. Although knowledge is gathered with respect to the dynamics and activity of individual components, it is still difficult to understand how all these

components interact with each other and work together to mount a protective immune response. Mathematical modeling of the intestinal immune system could help in understanding the dynamics of this complex biological system. Such a model should represent the major components of the system and their mutual quantitative relationships. The objective of this study was to construct a dynamic mathematical model of the cellular branch of the intestinal innate and adaptive immune system. We focus on the dynamics of early immunological development and the responsiveness to an infection with an invasive *S.* Enteritidis strain immediately after hatch.

7.2 Model development

Model description

A mathematical model was constructed describing the time course of early cellular immune development in the intestine in non-infected chickens, and of cellular immune development in the intestine during a *S.* Enteritidis infection. The model represents processes in the intestinal tissue, excluding the processes taking place in the lumen of the gut. Furthermore, the model addressed jejunal tissue which harbors different immune cells, including intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs). These different cell types in the intestine interact with each other, via intercellular communication. A schematic overview of all variables, their corresponding reactions, and influences of other cells, for non-infected and *S.* Enteritidis infected chicken is given in Figures 7.1A and 7.1B, respectively. For interactions between cell types identified which can be characterized as a saturating process, or as an active process that follows the law of enzyme kinetics, a Hill or Henri-Michaelis-Menten (HMM) functions were used.

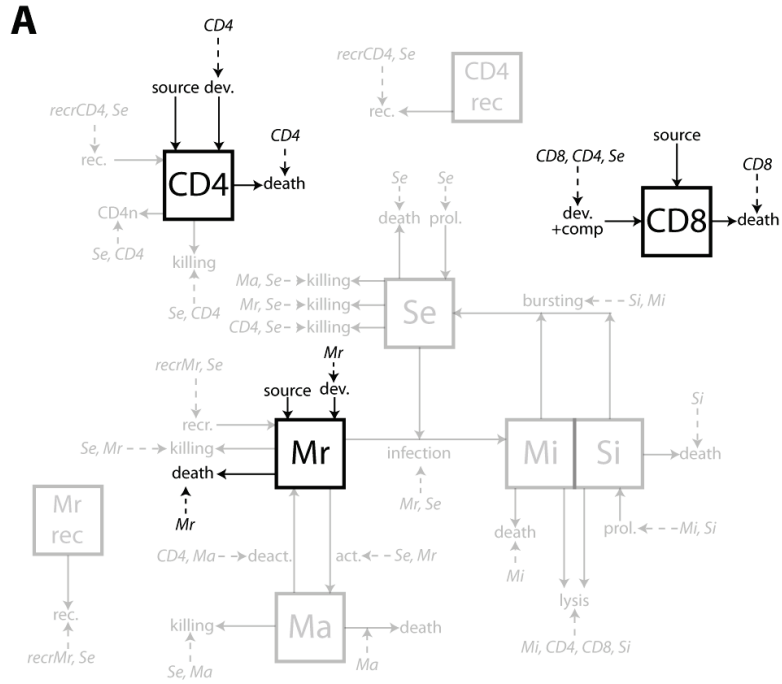
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Figure 7.1 (continued) Schematic representation of variables and their fluxes
In non-infected developing chicken (A), resting macrophages (*Mr*), *CD4*$^+$ and *CD8*$^+$ cells increase over time by different mechanisms. For these cells a fixed source (inflow) and death rate (outflow) per time was assumed. Moreover, the process of development (dev.) was described to account for the major changes in developing chicken. In *S.* Enteritidis infected developing chicken (B), the same components as in the non-infected chicken were present, but due to the *S.* Enteritidis infection, recruitment (rec.) of *CD4*$^+$ occurred from a basin outside the intestine (*CD4rec*). Furthermore infection of *Mr* by extracellular *Salmonella* (*Se*) occurred which consequently become infected macrophages (*Mi*) harbouring intracellular *Salmonella* (*Si*). These Mi can burst or undergo lysis, which respectively contribute to the amount of *Se* or clearance of *Se*. Due to the *S.* Enteritidis infection Mr is activated and become activated macrophages (*Ma*), and vice versa where Ma will be deactivated and become *Mr*. Together Ma and Mr will actively kill Se, as well as (naïve) *CD4*$^+$ cells. The *Se* and *Si* population have a proliferation (prol.) and death rate. Furthermore, due to the *S.* Enteritidis infection the increase of *CD8*$^+$ cells is negatively affected by *CD4*$^+$. Solid lines indicate fluxes, whereas dashed lines indicate which variables have an influence on that specific flux.

Macrophage related processes

The model for non-infected chicken, only included resting macrophages (*Mr*). The following processes involving macrophages were represented; a source, death and a developmental boost of *Mr* (Equation 1). Source is an influx of *Mr* from outside the intestinal system, which is estimated to be close to one per cent of the initial number of macrophages (Eq.1, *sMr*; 300,000 #Mr/(ml·d)). The death rate of Mr (Eq.1, *drMr*; 0.011 d^{-1}) is assumed to be in the same range as the death of macrophages in healthy adult mice or humans [21, 22]. In addition, we used a developmental boost for *Mr*, based on a law of population growth, with growth rate *devbMr* (Eq.1; 1.2 d^{-1}) and carrying capacity *kMr* (Eq.1; 2.5E+7 #Mr/ml).

In infected chicken, *S.* Enteritidis breaches the intestinal barrier and *Mr* encounter and phagocytize extracellular *S.* Enteritidis (*Se*). Subsequently, infection of macrophages (*Mi*) or activation of macrophages (*Ma*) occurs, the infection reaction (*Mr* to *Mi*) is expressed by a HMM equation for *Se* (Eq.1, 5; *iMi*, 0.1 d^{-1} [23]). Where the half-saturation of Se on infection Mr (*cSeMri*) is 600,000 #Se/ml. Similarly, the activation rate of macrophages, *Mr* to *Ma* (*aMa*, 100 d^{-1}), was described by an HMM equation (Eq.4), with an half saturation *Se* on activation *Mr* (*cSeMra*) of 1 #Se/ml. The *Se* are actively cleared by *Mr* and *Ma* in order to decrease the severity of infection. This clearance rate was defined by separate equations for *Mr* (Eq.1 and Eq.6, *kSeMr*; 5e-8 ml/(#Mr·d) [24]) and *Ma* (Eq.4 and Eq.6, *kMa*; 2.5e-7 ml/(#Ma·d) [24]) and their interaction with *Se* [24]. The rapid influx of macrophages, which is different from the developmental boost, is represented by a basin of macrophages (*Mrrec*) from outside the intestine. The corresponding reaction is expressed by a HMM equation with maximum velocity of *vrecMr* (Eq.1

and Eq.9; 1 #Se/(ml·d·#Mr) and Michaelis-Menten constant of *kmrecMr* (Eq.1 and Eq.9; 1,000 #Mr/ml). It was demonstrated that *S.* Enteritidis can survive within macrophages and is able to spread systemically in the host [25]. The representation of intracellular growth of *S.* Enteritidis is described in section '*S.* Enteritidis related processes'. The *Mi* are targets for lysis by the immune system, and *Mi* are eliminated via apoptosis by $CD4^+$ cells at a maximal rate of *lMi* (Eq.5; 0.8 d^{-1} [26]). Furthermore, both $CD4^+$ and $CD8^+$ cells ($CD8^+$) account for the cytotoxic effects, and this effect depends on the ratio of Total T cells ($CD4^+$ + $CD8^+$) and *Mi*, which was half maximal when the ratio is equal to the parameter *cCD4CD8* (Eq.5; 10 ($CD4^+$ + $CD8^+$)/*Mi*) [24]). Also we assume that *S.* Enteritidis is capable of opposing death of its host cell, which is denoted by $1 - papop\left(\frac{Si}{Si+N\cdot Mi}\right)$, where *apop* (Eq.5, 6, and 7; 0.7 (scalar) [24]) stands for the maximal per cent effect of intracellular *S.* Enteritidis (*Si*). *Si* is able to proliferate and if this proliferation is uncontrolled, the limit of the capacity N (Eq.5, 6, and 7; 30 Si/Mi [27, 28]) of the *Mi* to sustain bacteria will be reached, i.e. the maximum multiplicity of infection (MOI). When this threshold is exceeded the macrophage will die and bacteria will be released in the extracellular matrix. This process is represented by a HMM equation and growth occurs at rate *bMi* (Eq.5, 6, and 7; 0.4 d^{-1} [29]). When the infection is less severe, *Ma* will be inactivated by $CD4^+$ cells which is programmed by an HMM equation with maximum conversion rate *daMa* (Eq.1 and 4; 40 d^{-1}) and Michaelis-Menten constant *cdaMa* (Eq.1 and 4; 3E+07 #CD4/ml). An overview of all parameters regarding *Mr*, *Ma* and *Mi* are given in Tables 7.1, 7.2, and 7.3, respectively.

Table 7.1 The model parameters definitions and values of resting macrophages

Parameter Name	Short	Eq.	Range	Value	Unit
source *Mr*	sMr	1	300,000	300,000	#Mr/(ml·d)
death rate *Mr*	drMr	1	0.011-0.03	0.011	d^{-1}
activation rate *Mr*	aMr	1, 4	100	100	d^{-1}
half-saturation Se on activation *Mr*	cSeMr	1, 4	1	1	#Se/ml
infection rate *Mr*	iMr	1, 5, 6	0.1-0.9	0.1	d^{-1}
half-saturation Se on infection *Mr*	cSeMri	1, 5, 6	600,000	600,000	#Se/ml
killing *Se* by *Mr*	kSeMr	1, 6	5E-08	5E-08	ml/(#·d)
development boost *Mr*	devbMr	1	1.2	1.2	d^{-1}
development boost carrying capacity *Mr*	kMr	1	2.5E+07	2.5E+07	#Mr/ml
development boost *Mr* s1	s1	1	65	65	ml/#Mr
development boost *Mr* s2	s2	1	100	100	ml/#Mr
Recruitment *Mr* v	vrecMr	1, 9	1	1	#Se/(ml·d·#Mr)
Recruitment *Mr* km	kmrecMr	1, 9	1000	1000	#Mr/ml

Abbreviations used: Eq., equation

Table 7.2 The model parameters definitions and values of activated macrophages

Parameter Name	Short	Eq.	Range	Value	Unit
death rate *Ma*	drMa	4	0.011-0.08	0.08	d^{-1}
killing Se by *Ma*	kSeMa	4, 6	2.6E-07	2.6E-07	ml/(#Ma·d)
deactivation rate *Ma*	daMa	1, 4	40	40	d^{-1}
half-saturation $CD4^+$ on deactivation *Ma*	cdaMa	1, 4	3E+07	3E+07	#CD4/ml

Abbreviations used: Eq., equation

Table 7.3 The model parameters definitions and values of infected macrophages

Parameter Name	Short	Eq.	Range	Value	Unit
carrying capacity of *Mi*	N	5, 6, 7	7-30	30	Si/Mi
bursting rate *Mi*	bMi	5, 6, 7	0.05-0.5	0.4	d^{-1}
HMM constant *Mi*	mMi	5, 6, 7	2	2	scalar
lysis rate *Mi*	lMi	5, 7	0.4-0.8	0.8	d^{-1}
half saturation (*CD4*$^+$+*CD8*$^+$)/*Mi* ratio on lysisMi	cCD4CD8	5, 7	5-20	10	(*CD4*$^+$+*CD8*$^+$)/Mi
max percentage inhibition of apoptosis	apop	5, 7	0.7	0.7	scalar
death rate *Mi*	drMi	5	0.011-0.03	0.011	d^{-1}

Abbreviations used: Eq., equation

CD4$^+$ cells related processes

The model for infected chicken includes a representation of the number of *CD4*$^+$cells because they influence the deactivation of activated macrophages and stimulate lysis of *Mi*. In normal development (non-infected chicken) these will die at rate *drCD4* (Eq.2; 0.016 d^{-1} [30, 31]) and renewal of cells occurs at rate *sCD4* (Eq.2; 490,000 #CD4/(ml·d)). Like for *Mr*, also for *CD4*$^+$ cells a developmental boost was assumed to describe a rapid development of the intestinal immune system in time. Again an equation based on the law of population growth was used, with maximum growth rate *devbCD4* (Eq.2; 0.19 d^{-1}) and carrying capacity *k1CD4* (Eq.2; 8.2E+7 #CD4/ml). Furthermore a Hill like equation was programmed to create a sigmoidal relation with a first gradual increase followed by a rapid increase in velocity of *CD4*$^+$ cells, by the use of the Hill coefficient *ndevbCD4* (Eq.2; 2 (scalar)) and ligand concentration producing half occupation of *k2CD4* (Eq.2; 8.7E+6 #CD4/ml).

In *S.* Enteritidis infected chickens an influx of *CD4*$^+$ cells is observed at 1 day post infection. Therefore we assumed that (naïve) *CD4*$^+$ cells also participate in the response against *S.* Enteritidis, which is also observed for *S. Typhimurium* infections in mice [32]. This participation is embodied in an equation where *CD4*$^+$cells kill *Se* with rate *kSeCD4* (Eq.2; 1e-9 ml/(#CD4·d)), and a Hill like equation to create a sigmoidal relation for the interaction between naïve *CD4*$^+$cells and *Se*. A Hill

equation was programmed wherein the initial gradual increase is slow and then increases rapidly, with Hill coefficient *ndCD4* (Eq.2; 8 (scalar)), ligand concentration producing half occupation of *kSedCD4* (Eq.2; 4,200 ml/(d·CD4)), and maximum rate of *ydCD4* (Eq.2; 0.4 d^{-1}). The rapid influx of *CD4*$^+$ cells, which is different from the developmental boost, is represented by a basin of *CD4*$^+$ cells (*CD4rec*) from outside the intestine. The corresponding reaction is expressed by a HMM equation, with a maximum rate of *vrecCD4* (Eq.2; 100 #Se/(ml·d·#CD4)) and Michaelis-Menten constant *kmrecCD4* (Eq.2; 1 #CD4/ml). An overview of all parameters regarding *CD4*$^+$ cells is presented in Table 7.4.

Table 7.4 The model parameters definitions and values of *CD4*$^+$ cells

Parameter Name	Short	Eq.	Range	Value	Unit
source *CD4*$^+$	sCD4	2	490,000	490,000	#CD4/(ml·d)
death rate *CD4*$^+$	drCD4	2	0.01-0.33	0.016	d^{-1}
development boost *CD4*$^+$ rate	devbCD4	2	0.19	0.19	d^{-1}
development boost *CD4*$^+$ carrying capacity	k1CD4	2	8.2E+07	8.2E+07	#CD4/ml
development boost *CD4*$^+$ n	ndevbCD4	2	2	2	Scalar
development boost *CD4*$^+$ kCD4	k2CD4	2	8.7E+06	8.7E+06	#CD4/ml
recruitmentCD4 from *CD4recr* km	kmrecCD4	2, 8	1	1	#CD4/ml
recruitmentCD4 from *CD4recr* v	vrecCD4	2, 8	100	100	#Se/(ml·d.#CD4)
interaction (naïve) *CD4*$^+$ with Se y	ydCD4	2	0.4	0.4	d^{-1}
interaction (naïve) *CD4*$^+$ with Se b	ndCD4	2	8	8	scalar
interaction (naïve) *CD4*$^+$ with Se kSE	kSedCD4	2	4200	4200	ml/(d·#CD4)
killing Se by *CD4*$^+$ k4	kSeCD4	2, 6	1e-9	1e-9	ml/(#CD4·d)

Abbreviations used: Eq., equation

CD8$^+$ *cells related processes*

The model includes *CD8*$^+$ cells because 90% of the intraepithelial lymphocytes are T-cells and 80% of those are *CD8*$^+$ cells. It is known that *CD8*$^+$ cells are involved in de clearing of *Salmonella* [33]. In normal development (non-infected chicken) *CD8*$^+$ cells die at rate *drCD8* (Eq.2; 0.001 d^{-1} [30, 31]) and renew from source at rate *sCD8 (*Eq.2; 430,000 #CD8/(ml·d)). Comparable to *Mr* and *CD4*$^+$ cells, also for *CD8*$^+$ cells a developmental boost was presumed due to the rapidly developing intestine,

represented by population growth law with maximum growth rate *devbCD8* (Eq.3; 1.44 d^{-1}) and carrying capacity *k1CD8* (Eq.3; 1.3E+7 #CD8/ml). Furthermore a Hill like equation was introduced to create a sigmoidal relation, with *k2CD8* (Eq.3; 4.7E+7 #CD8/ml) as ligand concentration producing half occupation. Although $CD8^+$ cells have a minor role in this model, $CD8^+$ cells do affect the lysis of *Mi* (Eq.5, 6 and 7). Moreover, in the immunohistochemistry data a down-regulation of *CD8* cells was observed when comparing infected chicken with non-infected chicken, which could be an indirect effect of *S.* Enteritidis. This competitive effect is represented by inhibition of the developmental boost of $CD8^+$ cells by $CD4^+$ cells described with a Hill function with *ncompCD4* as the Hill coefficient (Eq.3; 0.5 (scalar)) and *kcompCD4* (Eq.3; 3.4E+7 #CD4/ml) for the ligand concentration producing half occupation, *compCD8* (Eq.3; 0.85 #CD8/ml) for the maximum rate of competition by $CD4^+$ and to ensure that this will only occur when *S.* Enteritidis is present, the following function was programmed Se/(Se+*w1*), where *w1* is 1e-25 (scalar) (Eq.3). An overview of all parameters regarding $CD8^+$ cells is given in Table 7.5.

S. Enteritidis related processes

S. Enteritidis stays either extracellular (*Se*) or intracellular (*Si*). The *Se* will proliferate within the intestinal tissue matrix with rate *pSe* (Eq.6; 35 d^{-1} [27]) and *Si* inside macrophages with rate *pSi* (Eq.6; 4.1 d^{-1} [34, 35]). The carrying capacity (*kSe*) for *Se* is set at 500,000 #Se/ml. Both *Si* and *Se* have death rates, respectively *drSi* (Eq.7; 0.05 d^{-1}) and *drSe* (Eq.6; 27.8 d^{-1} [36, 37]). An overview of all parameters regarding *Se* and *Si* is given in Table 7.6.

Table 7.5 The model parameters definitions and values of *CD8*$^+$ cells

Parameter Name	Short	Eq.	Range	Value	Unit
death rate *CD8*$^+$	drCD8	3	0.001-0.33	0.001	d^{-1}
source *CD8*$^+$	sCD8	3	430,000	430,000	#CD8/(ml·d)
development boost + competition *CD8*$^+$ a8	devbCD8	3	1.44	1.44	d^{-1}
development boost + competition *CD8*$^+$ k1CD8	k1CD8	3	1.3E+07	1.3E+07	#CD8/ml
development boost + competition *CD8*$^+$ k2CD8	k2CD8	3	4.7E+07	4.7E+07	#CD8/ml
development boost + competition mCD4 ncompCD4	ncompCD4	3	0.5	0.5	scalar
development boost + competition *CD8*$^+$ kcompCD4	kcompCD4	3	3.4E+07	3.4E+07	#CD4/ml
development boost + competition *CD8*$^+$ compCD8	compCD8	3	0.85	0.85	#CD8/ml
development boost + competition CD8 w1	w1	3	1e-25	1e-25	scalar

Abbreviations used: Eq., equation

Table 7.6 The model parameters definitions and values of extracellular and intracellular *Salmonella*

Parameter Name	Short	Eq.	Range	Value	Unit
death rate *Se*	drSe	6	25-35	27.8	d^{-1}
proliferation rate *Se*[a]	pSe	6	25-35	35	d^{-1}
carrying capacity *Se*	kSe	6	500,000	500,000	#Se/ml
death rate *Si*	drSi	7	0.05	0.05	d^{-1}
proliferation rate *Si*	pSi	7	4.08-7.92	4.1	d^{-1}

[a]Proliferation rate is estimated by the following formula: growth rate constant = ln(2) / 'doubling time', e.g. *Salmonella* doubling time is 30 minutes or, 1/48=0.021 per day, thus growth rate is approximately 33 per day.
Abbreviations used: Eq., equation

Model simulation software

The model was programmed in Complex Pathway Simulator (COPASI v4.6.33) [38]. Infections were simulated with the following time course: a duration of the simulation of 42 days and a simulation interval of 0.01 day. Because we lack day 0 immunohistochemistry measurements, realistic initial values of all state variables had to be estimated for this time point (Table 7.7). The deterministic LSODA method was used to calculate a time course. This numerical routine solves systems dy/dt = f (t,y) with a dense or banded Jacobian when the problem is stiff, but it

automatically selects between non-stiff (Adams) and stiff (BDF) methods. It uses the non-stiff method initially, and dynamically monitors data in order to decide which method to use. The parameter settings for the deterministic LSODA were as followed: 'integrate reduced model' was zero, which instructs COPASI to determine all variables through ODEs. A relative tolerance value of 1e-6 was used which indicates the level of accuracy of the numerical integration of the ODEs as a relative value. Likewise an 'absolute tolerance' value of 1e-12 was used for the absolute level of accuracy of numerical integration. The maximum number of iterative calculation steps was set to 10,000 for every integration step.

Table 7.7 Initial values of the variables for the model

Variable Name	Eq.	Common Name	Non-infected (#/ml)	Infected (#/ml)
$CD4^+$	1	$CD4^+$ cells	9e+06	9e+06
$CD8^+$	2	$CD8^+$ cells	7e+06	7e+06
Mr	3	Resting macrophages	9e+06	9e+06
Ma	4	Activated macrophages	0	0
Mi	5	Infected macrophages	0	0
Se	6	Extracellular *Salmonella*	0	200
Si	7	Intracellular *Salmonella*	0	0
CD4rec	8	Basin for recruitment $CD4^+$	2.7e+07	2.7e+07
Mrrec	9	Basin for recruitment *Mr*	2e+07	2e+07
TotalM	10	Sum of *Mr*, *Ma*, and *Mi*	9e+06	9e+06
TotalT	11	Sum of $CD4^+$ and $CD8^+$	1.6e+07	1.6e+07
TotalS	11	Sum of *Se* and *Si*	0	200

Abbreviations used are Eq., Equation.

Model simulation software

Half of a group of one-day-old chickens were infected with *Salmonella enterica* serovar Enteritidis [39]. The details of the experimental design are described by Schokker *et al.* (2009, 2010a). Chicken were inoculated orally with $1 \cdot 10^5$ *S.* Enteritidis in 0.2 ml Phosphate Buffered Saline (PBS) or with 0.2ml PBS at day 0. Intestinal samples were taken at, 8 hours, and 1, 2, 4, 8, 12, and 21 days post hatch or infection. For each time point 10 chickens of each group were used. The bacteriological data used in the model are the number of colony forming unit per milliliter of *S.* Enteritidis bacteria in the liver.

For immunohistochemical examination the intestinal samples were snap frozen in liquid nitrogen and used to make cryosections. Similar to Schokker *et al.* (2010a), slides (8 µm) were incubated with monoclonal antibodies against chicken $CD4^+$ cells (1:200 diluted; CT-4, Southern Biotech), or macrophages (1:50 diluted; KUL-01, Southern Biotech), next to the $CD8^+$ (CT-8, 1:200, Southern Biotech) staining. To estimate the amount of cells in 1 milliliter tissue (Figure 2), the following calculation was performed: first the number of positive stained cells per mm^2 was calculated for each slide with the use of Image-Pro Plus (version 6.2, Media Cybernetics); second this number was multiplied by 125 to get the number of cells per square millimeter; thirdly, the latter number was multiplied by 1,000 for the number of cells per cubic milliliter; finally, an average for each time point and cell type was calculated.

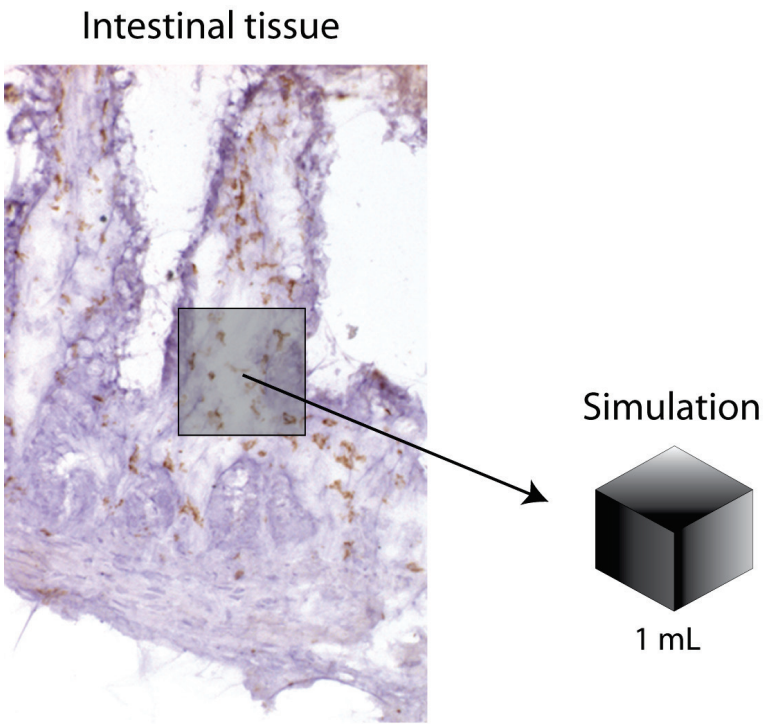

Figure 7.2 Model assumptions
The biological system that was modeled involves a cross section of non-infected jejunal tissue (colored purple) four days post hatch including macrophages (colored brown). Data used for the present modeling study were derived from a jejunal tissue preparation of approximately 1 milliliter.

Model evaluation

Different datasets were used to evaluate model prediction in non-infected chicken. These datasets were obtained from different chicken lines in different studies: 1) a fast growing [15] and 2) a slow growing chicken line [15]; 3) chicken line B [40] and 4) chicken line C [40]; 5) a chicken line A [41]; 6) a chicken line D (unpublished). Four out of these six studies also delivered data that could be used to evaluate the model output due to an infection with *S.* Enteritidis: two datasets by [15]; and 2 datasets by [40].

7.3 Model evaluation and simulation runs

Parameter estimation

Not all required parameter values or equations were available. Therefore, parameters were optimized to represent the data obtained from this chicken line A or from literature (Tables 7.1-7.6). Although most of the parameter values were derived from literature based on the data of adult mice instead of chicken, we anticipate that they are reliable with respect to the order of magnitude of these parameter estimates.

Model behavior

Figures 7.3 and 7.4 represent the result of simulation runs from hatch (day 0) to day 42. Figure 7.3 shows the predicted model outputs together with the immunohistochemical data of non-infected chicken. Figure 7.4 shows the predicted model output together with the immunohistochemical data of infected chicken. After calibration with the immunohistochemical data, the model curve of non-infected chicken represents the biology accurately for all three variables. Thus, the selected cell types for our model and their corresponding relationships were able to portray the biology in early intestinal development. For *S.* Enteritidis infected chicken, the curves of $CD4^+$ cells and *S.* Enteritidis appear to correspond with observed experimental values. However the curves for $CD8^+$ cells and macrophages reflected the experimental data less well. The rapidly changing dynamics of $CD8^+$ cells was not captured by this mathematical model. Similarly, under experimental conditions the number of macrophages peaked at 4 days post infection and subsequently dropped to a stable level, whereas in the model predictions the macrophage peak was too high and returned too slow towards homeostatic values. To assess to what extent these two models reproduce observed values, the R-squared values were calculated for each variable (Table 7.8). For non-infected chicken the R-squared values are all above 0.83, meaning that only 17 per cent of the experimental observations remained unexplained by the model. For the *S.* Enteritidis infected chicken $CD4^+$ cells have the highest R-square of 0.84, followed by a R-square of 0.49 and 0.43 for Se and $CD8^+$ cells, respectively. The score for macrophages was only 0.26, due to the fact that the model could only reproduce the time course of development of macrophages at 8 hours to 2 days post hatch.

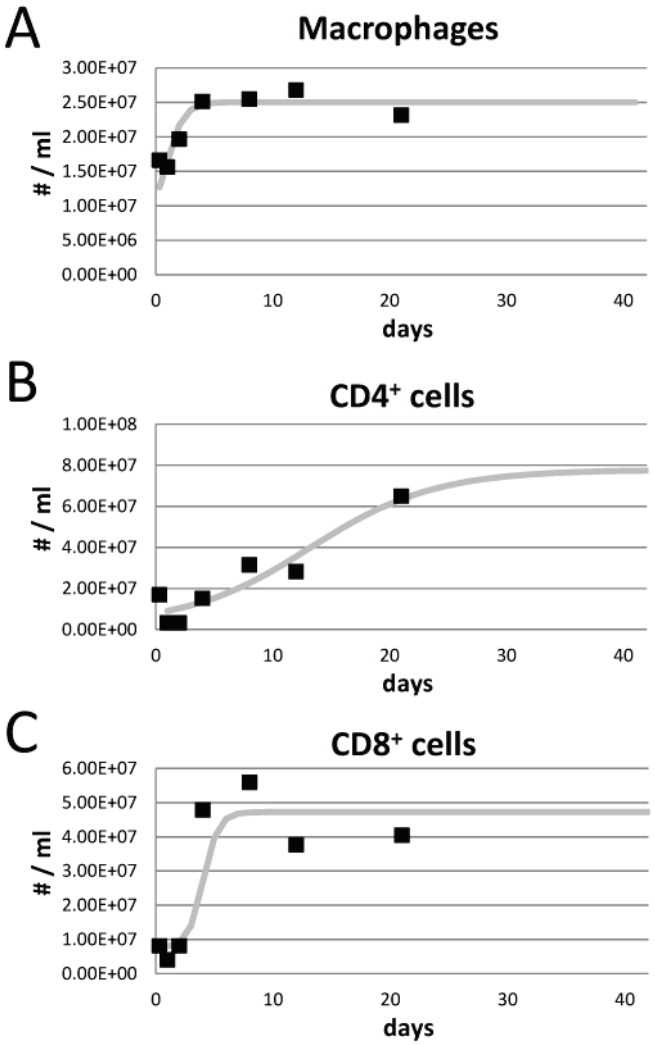

Figure 7.3 Comparison of model prediction with data of non-infected chickens
In all graphs, the horizontal axis depicts time in days post infection and the vertical axis the number of cells per milliliter in intestinal tissue. Grey curves depict the model prediction, whereas black symbols indicate the average of the observed immunohistochemistry data (max. 10 chicken, 3 observations per chicken). A, depict macrophages; B, *CD4*$^{+}$ cells; and C, *CD8*$^{+}$ cells.

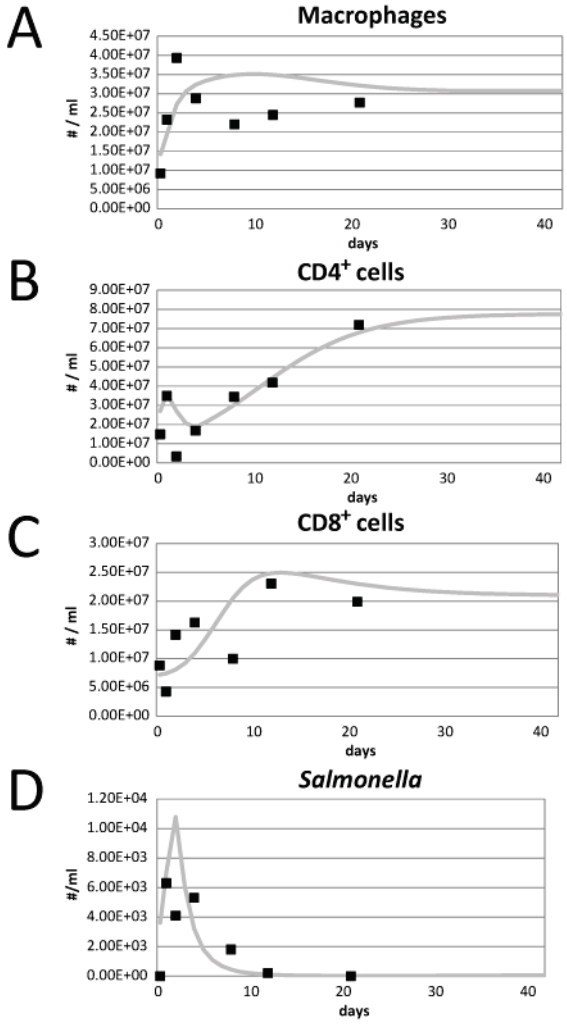

Figure 7.4 Comparison of model prediction with data of infected chicken
In all graphs the horizontal axis depicts time in days post infection and the vertical axis the number of cells per milliliter in intestinal tissue. Grey curves indicate the model prediction, the black symbols indicate the average of the immunohistochemistry data (max. 10 chicken, 3 observation per chicken). A, depict macrophages; B, *CD4*$^{+}$ cells; C, *CD8*$^{+}$ cells, and D, *Salmonella*.

Table 7.8 R-Squared values of predicted curve versus real data

Cell Type	Non-infected	Infected
Macrophages	0.83	0.26
CD4^{+} cells	0.90	0.84
CD8^{+} cells	0.88	0.49
S. Enteritidis	NA	0.43

Abbreviations used: NA, Not available

Model evaluation

The model was evaluated against cellular immune data of other studies which were not used to setup the model. Hereby, we investigate the robustness of the model and the impact of genetic background of the chicken line on prediction accuracy for immune development and response to infection. From the results shown in Figure 7.5, it can be concluded the model probably was made chicken-line or chicken-experiment specific. In an early stage of intestinal development, observed values were similar for all data sets and comparable to model predictions. However, at later stages of intestinal development (day 4-21) the model output started to deviate increasingly from observed biological values. For 21 to 42 days post hatch no external data points were available. When plotting the average of the immunohistochemical data for each of the cell types in time, a high variation between experiments and between chicken lines was observed which complicates model evaluation (Figure 7.6).

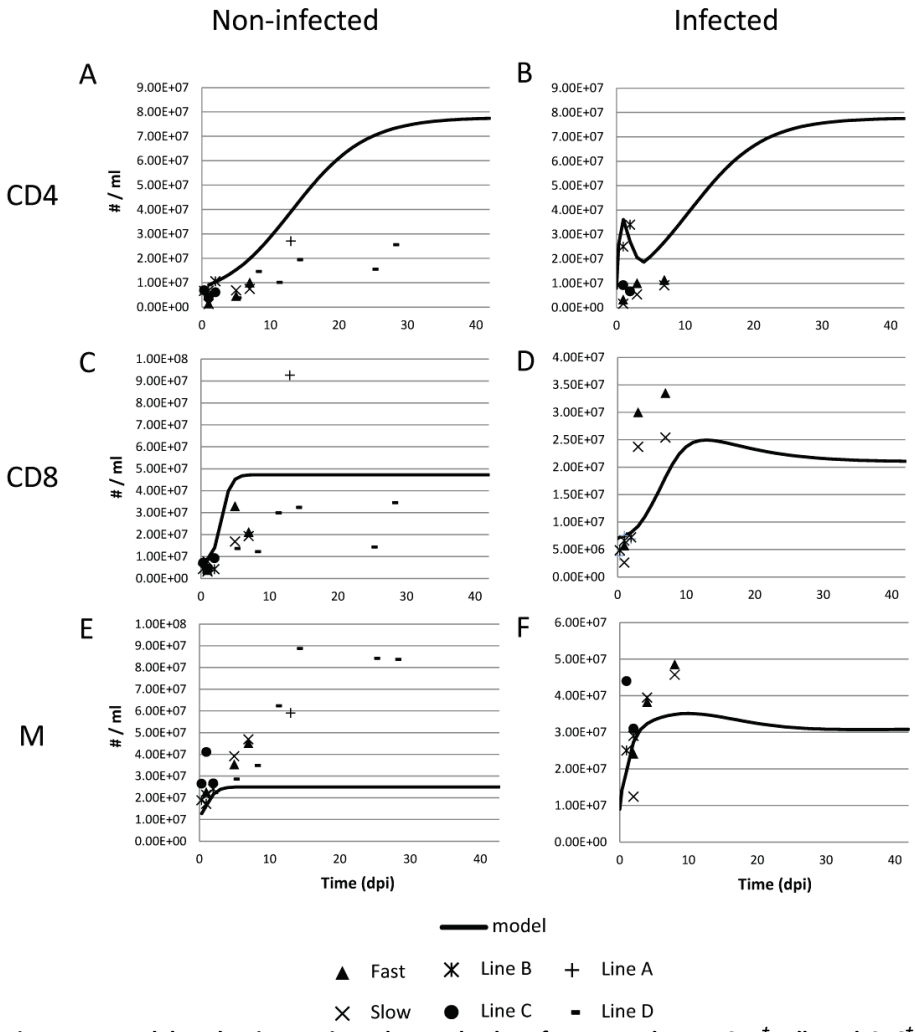

Figure 7.5 Model evaluation against observed values for macrophages, *CD4*$^+$ cells and *CD8*$^+$ cells in both non-infected and infected chicken

For all experiments the number of cells is plotted against time in days post infection. Macrophages (M): A, non-infected; and B, infected chicken, *CD4*$^+$; C, non-infected; and D, infected chicken, and *CD8*$^+$: E, non-infected; and F, infected chicken. Datasets of the following lines and/or studies are included for non-infected chicken: 1) fast growing [15] and 2) slow growing chicken lines [15]; 3) chicken line B [40] and 4) chicken line C [40]; 5) chicken line A [41]; and 6) chicken line D an unpublished study. Whereas for *S.* Enteritidis infected chicken only 4 out of these 6 studies were available, including the two datasets of [15] and the two datasets of [40].

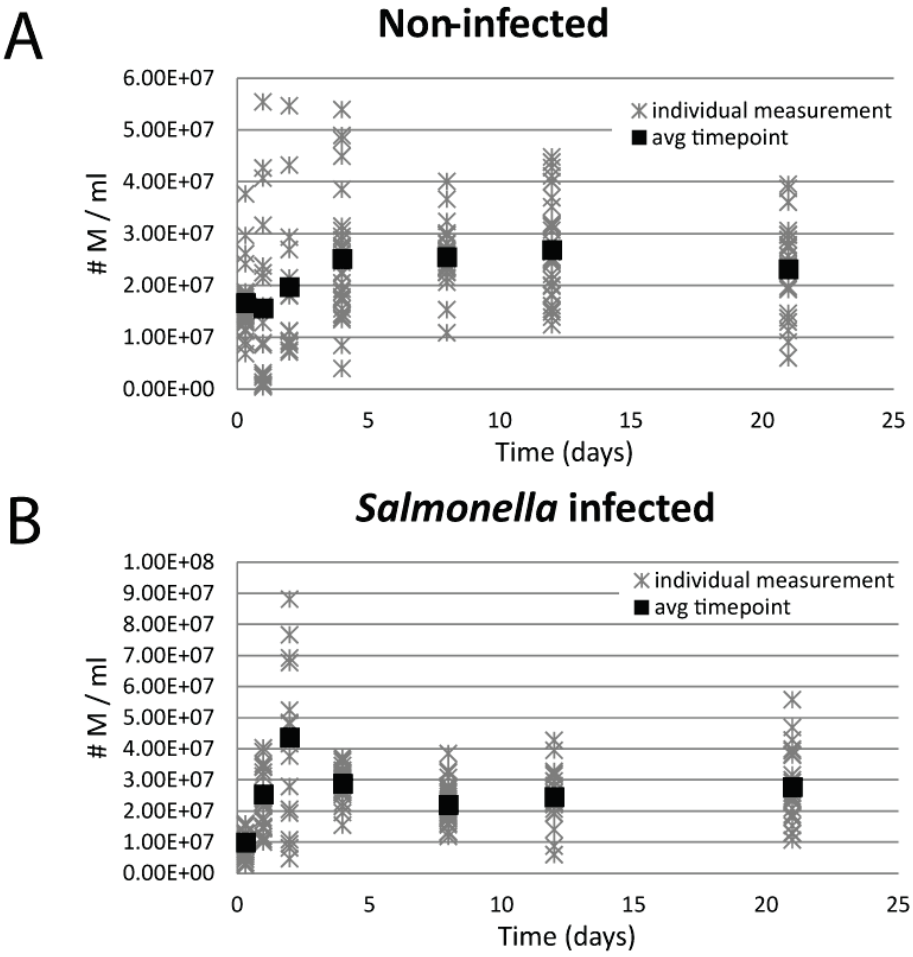

Figure 7.6 Biological variation between chicken

Variation in immunohistochemistry data of macrophages (M) in both non-infected (A) and *S.* Enteritidis infected chicken (B). On the vertical axis the number of macrophages per milliliter is depicted and on the horizontal axis the time in days post infection. Observations for individual measurements are depicted by a grey asteriks, whereas the average per time point is depicted by a black square.

7.4 Discussion

In previous work we investigated the dynamics of intestinal immune development at the gene expression level for both non-infected [1] and *S.* Enteritidis infected chickens [42]. From these studies we concluded that the well-orchestrated spatiotemporal pattern of immune development in non-infected chickens is considerably disturbed by an oral infection with *S.* Enteritidis. Although these studies provided insight into the dynamics of immune related processes in intestinal tissue, they did not provide insight into the dynamics of cellular immunity. Furthermore, immunohistochemical measurements do not provide enough information on the mutual interactions in time between cellular components of the intestinal immune system and the response dynamics against invading pathogens. This was the main driving force behind our initiative to start the construction of a dynamic mathematical model of the cellular branch of the intestinal innate and adaptive immune system of chicken from day 0-42 after hatch. Availability of a mathematical model may provide more understanding of the mutual interaction between the cellular components of the immune system and explain the behavior of the system in response to external stimuli. Such a model provides the opportunity to run simulations to test different conditions and variation in input parameters without the need of animal experimentations.

Modeling non-infected development

Since we choose to include developmental aspects, we introduced into the model a developmental boost for *Mr*, $CD4^+$ and $CD8^+$ cells. The developmental boost equations represent the biological phenomenon that after hatch the intestine, including the intestinal immune system, develops very rapidly [2, 3, 43]. We assumed that the small areas of the jejunum, which were used for immunohistochemical analysis, are representative for the whole jejunum tissue. To minimize the technical variation of the immunohistochemical measurements, we counted intestinal slides of a maximum of ten individual chicken per time point and for each intestinal section we calculated the average of three counted areas. Although the chicken were already selected based on similar weight and numbers of ceacal secreted *Salmonella*, variation in the number of cells per milliliter between chickens was still high (Figure 7.6). This high variation could be due because it was not possible to follow the same chicken in time, because only after sacrificing the animals it was possible to obtain immunohistochemical values. Moreover the variation between chickens was probably enlarged by converting the two dimensional data to three dimensional data (from square centimeters to

cubical centimeters) and hence the deviated prediction of this model in relation with the biological data may have a methodological cause as well.
This transformation was necessary to ensure that both the bacteriological and immunohistochemistry data were expressed per unit of tissue volume. To investigate whether the assumed interactions and accompanying variable values were plausible, the model output was plotted against the average of the immunohistochemistry data of the non-infected chicken and subsequently the R-squared values were calculated (Figure 7.3 and Table 7.8). These results indicated that the selected nodes and their relationships were accurately represented in the model.

Modeling S. Enteritidis infection during development

S. Enteritidis colonizes the intestine and (trans)migrates to the spleen, liver and other tissues. We assumed that the same percentage of the pathogen inoculum crosses the intestinal barrier in the young chicken as observed in adult mice [44], which is approximately 0.05% of the inoculum. Thus, approximately 200 bacteria will enter the 1 cm^3 system at day zero. Furthermore, we assumed that the number of CFUs in the liver [45] is representative for the amount of extracellular S. Enteritidis present in the entire intestinal tissue. The host evokes an immune response against the intracellular and extracellular S. Enteritidis in the intestinal tissue. Extracellular S. Enteritidis will be phagocytosed by macrophages, but this pathogen can survive within these cells [25]. Phagocytosis an migration of macrophages results in a systemic spread of *Salmonella* in the host. The severity of a systemic infection depends on a variety of factors such as the immune response, barrier functions, genetic background of the host, and the genetic background of the *Salmonella* [40]. Clearance of an infection is dependent on the immune system, where time and efficacy of the immune response are of importance. Active recruitment of macrophages and the involvement of $CD8^+$ cytotoxic T cells in the immune response against *S. typhimurium* [46] suggests a T helper 1 response, and we expect a similar response to S. Enteritidis. Previous studies showed that the ability to transfer protective immunity to virulent *S. typhimurium* is impaired by exhaustion of either $CD8^+$ or $CD4^+$ cells [47-49] meaning that both cell types are of importance in host response against *Salmonella*. Therefore the focus in the current study was primarily on macrophages, $CD4^+$ and $CD8^+$ cells as elements of the immune response, because B cells are of no or less importance for clearance of the pathogen [50].
To investigate to what extend our assumptions and reactions in the model representing the infected status were correctly defined, R-squared values were

calculated for macrophages, *CD4*$^{+}$, *CD8*$^{+}$ cells, and *Salmonella*. The model output of macrophages did not accurately describe the experimental data, which suggest that processes from outside the system were misrepresented and/or neglected. Cytokines are good candidates for this omission because they have important functions in the regulation of the cellular immune response. It could be that the addition of cytokines into the model will improve the fit with macrophage dynamics. Processes regarding *CD4*$^{+}$ cells were correctly incorporated into the model, because the R-square was 0.84, meaning that most of the variation is captured by the model. The experimental data of *CD8*$^{+}$ cells showed rapid fluctuations, which could not be captured by the model. The R-square was only 0.49, but the model was able to generate a similar trend as observed in the experimental data. The model predicted that *Salmonella* was cleared at approximately 6 days post infection, while the biological data showed clearance at 12 days post infection (Figure 7.4; R-square 0.43, Table 7.8). This difference between the model and the experimental data indicates that the interactions and relationships of macrophages were not accurately modeled.

Model application and future model development

This mathematical model can be used to simulate the course of a *S.* Enteritidis infection in the chicken intestine with a varying initial number of immunological cells, or with another set of parameter values for recruitment rate, proliferation rate, or death rate based on the genetic background of the chicken. This model was calibrated with one chicken line, and clearly showed that changing parameter values is necessary to be able to simulate genetically different chicken lines. Multiple scenarios can be run, after parameterization for different chicken lines or different Salmonella strains, and subsequently the outcome can be monitored. For example, by changing the initial number of *Mr*, it seems possible to reproduce the observations for a genetically different chicken line, namely line C which has a higher number of macrophages at hatch. When doubling the initial number of *Mr* at hatch, the severity of a systemic infection decreases with approximately one order of magnitude (Figure 7.7), which is in line with the experimental data. This is one example which shows how the model can be used for testing the effect of different variables on model output, i.e. severity of infection.

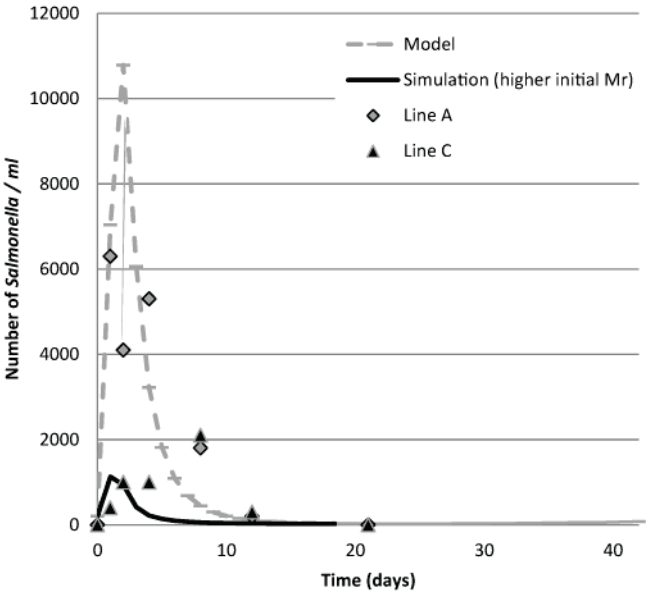

Figure 7.7 Prediction of the effect of an increase in initial number of macrophages
The x-axis depicts time after infection in days, whereas the y-axis depicts the number of *Salmonella* per milliltre. The curves depict predicted (grey, dashed) dynamics of the number of *Salmonella* in time by the model, and predicted dynamics when starting with a a higher number of macrophages at hatch (black, solid). In addition, experimental data are plotted for two genetically different chicken lines: line A on which the model was calibrated (grey diamond); and line C which represents the case study which had an increased initial number of macrophages (black triangle).

Conclusions

We have set the first step towards the development of a mathematical model representing the dynamics of major phenomena of cellular immune development of both the innate and adaptive immune system in young non-infected chicken and in chicken infected with *S.* Enteritidis. Quantitative experimental data were accurately represented in the model for non-infected animals and to a lesser extend for infected animals. Although the model was calibrated on a single chicken line, it was possible to simulate the behavior of other genetically different chicken lines. Model simulations have shown that this mathematical model has the potential to describe the effect of different input variables.

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8

General Discussion

8.1 Introduction

Optimal intestinal health and functionality is a prerequisite for sustainable animal production. Investigating the functioning of the intestine as a system is instrumental to understand how the various, diverse, and closely connected processes in the intestine are influenced by internal and external factors. In this context it is important to know which processes are robust, which are flexible, which are most susceptible for modulation, and how and by which internal (e.g. hub genes) and external (e.g. feed components) factors adaptations of the intestine are orchestrated. The intestine is a complex tissue harboring dynamic ranges of multiple cell types that strongly interact and communicate with each other and which are influenced by feed and microbial constituents in the intestinal lumen. Previously, specific aspects of intestinal health and functioning have extensively been studied by different approaches. The recent developments in the areas of genomics and computational sciences now provide us with tools and methods to start studying the behavior of biological systems as a whole. In the research described in this thesis we applied genomics and computational approaches to describe (molecular) processes that are associated with two contrasting physiological conditions of developing intestinal tissue of young growing chicken. We used an infection with *Salmonella* immediately after hatch and control animals to create the two contrasting physiological conditions. In Chapters 2-7 we applied different genomic and modeling approaches to get a global view of the physiological activity of the intestine and to identify and describe the major components and processes and their (quantitative) relationships involved in the functioning of the system of the gut under the two contrasting conditions. In this Chapter, I will describe processes and high level regulators that potentially drive the development and adaptation of the gut-system under normal conditions (section 8.2) and under disturbed conditions (section 8.3). A comparison between the two physiological conditions provides information on the mechanism used by the tissue to adapt to environmental changes. I will describe processes highly susceptible to the experimentally induced physiological changes and processes that seem to be rather independent of these changes. I will also critically discuss the different methods used for the research described in this thesis, the current challenges related to network validation, the advantages and disadvantages of mathematical modeling (section 8.4) and how to improve the predictability of the mathematical model (section 8.5). Furthermore I will point towards new research ideas and objectives with the use of -omics data in relation to intestinal functioning

and health, and towards potential application of the knowledge generated in this research (section 8.5).

8.2 Intestinal Development

The transcriptomics approach that was applied in this research generates quantitative genome-wide expression data of genes. Gene expression is the most fundamental level at which an organism's genotype, in interaction with its environment, dictates its phenotype. Therefore the genome-wide transcription profiles generated in these studies may be considered to represent the physiological status of the intestinal tissue at the time of sampling. In contrast to most other transcriptomics studies, we extracted samples over time, from 8 hours post hatch to 21 days post hatch, allowing to perform time-series analyses. With these analyses we identified temporal gene expression patterns (Chapter 2). In fact, such an analysis generates a kind of movie showing gene expression changes of intestinal tissue during the sampling period. Since the sampling started at 8 hours post hatch, the chosen sampling period provides insight into the early developmental changes of the intestine. Functional analysis of the cohort of genes residing in each 'expression pattern' cluster as described in Chapters 2 and 3 indicated that each cluster could be categorized in one of the three dominant processes of intestinal development (as defined in the general introduction), namely morphological, functional, and immunological development. I hypothesize that genes belonging to one cluster are involved in closely related processes, including the genes and/or probe sets which are still un-annotated. The time series analysis clearly illustrated that genes involved in the various processes, like morphological processes, are expressed in a well-timely-ordered fashion. Genes involved in functional processes are moderately expressed 8 hours to 2 days after hatch, which could suggest that the first feed intake activates the expression of genes involved in such functional processes. The expression level of genes involved in these functional processes decreases in time, which could be due to the differentiation of cells and the optimization of functional processes that occur during development. Genes involved in morphological processes are highly expressed immediately after hatch followed by decreasing expression levels over time. These genes are higher expressed compared to functional related genes, which suggests that immediately after hatch morphological processes dominate intestinal development. This is logical because the typical intestinal morphological structure and surface expansion need to be established before functional processes can take place efficiently. In fact it is known from morphological studies that during

that period the intestine develops rapidly, crypts are formed and villi length, width, and density increase [1]. Genes involved in immunological development show an expression pattern with relatively low expression immediately after hatch and increasing expression over time. I hypothesize that this gene expression pattern reflects the timing of the colonization of the gut by microorganisms and the subsequent microorganism-mediated induction of immune development [2]. This is supported by the immunohistochemistry data obtained for CD4+, CD8+, TCRαβ+, TCRγδ+ and KUL01+ (e.g. macrophages) cells in the same time-series intestinal tissue samples (Chapters 3 and 4). The general trend of these immune cells is that their number increases over time which runs parallel to the expression profiles of genes involved in immunological development. During the sampling period, genes involved in turnover processes showed expression levels close to baseline throughout the time-series, suggesting that no significant changes in turnover processes occur from day 1-21. These turnover processes probably represent the continuous apoptosis and proliferation of epithelial cells. This suggests that replacement of epithelial cells is a development-stage independent process which is already in full operation at the day of hatch.

It should be stressed here that the datasets are generated from *in vivo* intestinal samples consisting of multiple cell types. Therefore the gene expression patterns provide a comprehensive view of the ongoing molecular processes in a complex and highly dynamic tissue. Another point of attention is that only a small section of the intestine, approximately 1 cm^3 of tissue, was taken for analysis. It could be argued that such samples do not represent the surrounding intestinal tissue, due to local effects. On the other hand, the functional information obtained with the datasets derived from these small tissue samples is supported by morphological and immunological observations in previous studies [2, 3].

The analysis of gene expression data as performed in Chapters 2-3 were focused on providing a better insight into the temporal development of functional processes in the gut rather than on the regulation of gene expression and its coherent phenotype. The best-studied level of gene regulation is at the DNA sequence level which involves promoters, enhancers, silencers, RNA polymerases, transcription factors, cofactors, and chromatin remodeling [4-7]. Gene regulation at the nuclear level involves the dynamic spatial organization of the genome inside the nucleus [8]. In Chapter 6 we investigated whether there exist other levels of gene regulation that operate at the tissue level by inferring gene association networks (GANs) from time-course mRNA expression data. GANs are very complex and difficult to interpret especially when such networks have high numbers of nodes and edges, and when they represent complex and dynamic cell populations.

Nevertheless the GANs show that gene associations exist which deviate from the associations seen in cluster and functional and/or pathway analyses. I hypothesize that the GANs as described in Chapter 6 provide an image of hitherto unknown regulatory mechanisms active at the tissue level. Regulation at the tissue level has recently also proposed by others [9]. The GANs follow a power law, i.e. scale-free network, which is observed for many biological networks and which suggest that the method is performing correctly [10]. By calculating the putative direction of the gene-gene interactions in the GAN, we found that the highly connected genes (hubs) influence their direct neighbors over time and not vice versa. Thus by modulating the expression of the hub genes by either internal or external stimuli, the expression of all the neighboring genes will be affected as well. A better understanding of these interaction networks and their relationship with physiological effects may help to discover how the behavior of complex biological systems can be modulated. For example, it may be possible to modulate intestinal functioning and health by influencing the expression of hub genes. Bioinformatic analysis also suggested that the identified hub genes are potential high level regulators of the major ongoing processes in the intestinal tissue as determined in Chapters 2, 3, and 4 and by more traditional research measurements [2, 3, 11].

In Chapters 2-6 we investigated the temporal development of functional processes in the gut and its potential high level regulation. A next step in system-based approaches, is to describe relationships at a higher biological aggregation level. Therefore we put efforts into the generation of a mathematical model representing a particular aspect of the intestine. As a first step we choose to focus on the cellular compartment of the immune system (Chapter 7), because this subsystem is well defined and highly dynamic, especially during intestinal development, and because it is relatively easy to generate quantitative data of its individual components. The generated model describes the temporal dynamics of the cellular components of the intestinal immune system. There is a good correlation between the model output and the real biological data used for parameterization of the model, since the R-square values ranged from 0.83-0.90. In addition the model output fitted with experimental data of early intestinal development of three different chicken lines. The high R-square values indicate that we determined both the selected cell types (nodes) and the assumed interactions (edges) more or less correctly. Nevertheless, because we had to introduce a developmental boost for macrophages, CD4+ and CD8+ cells, I anticipate that the model does not capture all details of early immunological development.

We started the modeling approach with the building of model frameworks that differed in complexity, i.e. the number of nodes. During the subsequent modeling process, these models were refined and a balance was found between model complexity and model solvability. From this modeling process I learned that by focusing on the major components of the system and their mutual interactions, it is possible to reduce the complexity substantially without major effects on the gross model output. The iterative process of mathematical modeling and fitting with experimental data is a useful activity for better understanding of the behavior of a system. Addressing a system in mathematical equations, broadens your scope and understanding of the system. For example, by expressing the influx of macrophages in equations, the velocity of this process can be closely monitored. Another example is that by performing model sensitivity analyses, the effect of parameters (i.e. biological processes) on the outcome of the system can be investigated.

8.3 Disturbed Development

Clustering of gene expression data followed by functional analysis learned that an infection with *Salmonella* in young chicks affects the timing of several developmental processes in the gut. In the disturbed chicken intestine morphological processes are delayed in time, which suggest that these processes are of less importance for the host during the adaptation to the infection with *Salmonella*. In contrast, metabolic processes are unaffected by *Salmonella* and still highly expressed after hatch as was observed during normal intestinal development. Apparently, functional processes are in this case more important than morphological processes. I hypothesize that a constant uptake of nutrients and energy supply are crucial factors for efficient system adaptations and that after a distortion with *Salmonella* energy supply is used for (immunologic) adaptation processes to the cost of morphological processes.

Based on general knowledge, we expected to find a clear and well-ordered change in the expression of immune related genes and a corresponding measurable immune response after the infection with *Salmonella*. To our surprise this well-ordered change in gene expression could not be detected (Chapter 2) although differences in cellular immunity could be detected (Chapter 3). The expression of genes involved in activation and recruitment of macrophages and T cells are not simultaneously induced after the infection with *Salmonella*. This could be due to the immaturity of the intestinal immune systems at the moment of infection. It could also be due to the experimental setup, because our focus was on temporal gene expression changes during an infection and only the *Salmonella* infected

samples were separately analyzed (i.e. control data were not loaded and analyzed together with the *Salmonella* data). Nevertheless, the expression of a number of immune related genes was affected by *Salmonella*, although their expression pattern did not cluster. An in-depth analysis of these immune related genes (Chapter 3) showed that in the *Salmonella* disturbed intestine, compared to control animals, a number of immune pathways are expressed earlier in time. This indicates that after an infection with *Salmonella* the intestinal systems adapts to the changing conditions by the immediate induction of immune related processes, although these processes do not dominate the global adaptation mRNA profile. The latter was not shown before and demonstrates the added value of systems approaches.

The work described in Chapter 4 shows that several intestinal gene expression clusters are positively or negatively correlated with the severity of a systemic *Salmonella* infection. It can be argued that positively correlated genes contribute to a reduced adaptation of the system, whereas negative correlated genes contribute to an improved adaptation. In other words, higher cell turnover and tissue repair processes are correlated with improved adaptations of the gut and result in reduced severity of systemic disease. The process of cell turnover is closely related to tissue repair, because in the intestine constant renewal and shedding of cells occurs. I hypothesize that active modulation of these processes is directly coupled to the integrity of the intestinal epithelium and therefore also to the ability of the gut tissue to adapt to the infection with *Salmonella*. Therefore, I suggest that intestinal cell turnover processes should be investigated more thoroughly, in order to identify genes and processes which can be modulated (externally or by genetics) to optimize intestinal functioning and thereby health. The positively correlated genes are mainly involved in immune related processes and wound healing. These processes are probably a direct effect of the damage caused by the *Salmonella* infection and important for the clearance of *Salmonella* and repair of epithelial tissue.

To investigate the role of genetics in intestinal development and adaptation mechanisms, we included three genetically different chicken lines (lines A, B, and C) in our studies (Chapter 5). Comparing the susceptibility of genetically different chicken lines is another approach of identifying components and processes that may contribute to efficient intestinal adaptation mechanisms. The results indicated that genetically different chicken employ different routes and processes to counteract an infection with *Salmonella*. According to bacteriological and immunohistochemistry data lines B and C appear to be more resistant at hatch,

compared to line A. From the data presented in Chapter 5 it can be concluded that intestinal barrier function is an important factor for efficient adaptation and resistance mechanisms against *Salmonella* infection. These findings are in line with the data described in Chapter 4. Thus not only immune related genes are candidate targets for the selection of *Salmonella* resistant chicken, also genes involved in (early development of) intestinal barrier function may be potential targets. Therefore, I suggest that besides intestinal cell turnover processes, intestinal integrity mechanisms should also be investigated more thoroughly, in order to identify genes and processes which can be modulated (externally or by genetics) to optimize intestinal functioning and thereby health.

Similar to the analysis of healthy chicken, we also inferred a GAN from longitudinal expression data of infected chicks. Although starting with the same set of genes, a completely different GAN topology was obtained. This suggests that an infection with *Salmonella* changes the global regulation of tissue behavior considerably. Remarkably, compared to the GAN of the control tissue, a completely different set of hub genes dominated the GAN representing the infected intestinal tissue. Only one out of the top twenty hubs was the same in both GANs. This suggests that depending on the external stimulus, other hubs and other gene-gene interaction are the driving force behind systems behavior. Bioinformatic analysis of the identified hubs (Chapter 6) suggested that the disturbance with *Salmonella* is associated with a shift from transcriptional regulation in the non-disturbed tissue to cell-cell communication in the disturbed tissue [9]. A number of these cell-cell communicator hubs are involved in immune responses, like IFNG which regulates up to 30 other immune related genes involved in the homing and adhesion of immune cells, the promotion of the Th1 response, and the increase of antigen presentation. Apparently, transcriptionally-regulated cell development and cell differentiation are major processes during normal development, whereas cell communication-based tissue remodeling and (immune) cell signaling are major processes after a disturbance with *Salmonella*. This shift in hubs and GAN topology seems logical because cells at the site of infection are signaling to initiate the homing and influx of immune cells and to initiate repair mechanisms, as observed in Chapters 3, 4, 7, and other studies. Besides the cell signaling hubs, the GAN representing the disturbed status also contained hubs related to developmental and metabolic processes. This is in agreement with our observation described in Chapters 3 and 4 that under the disturbed conditions processes related to morphologic and functional development are dominant. The added value of the GAN approach is the visualization of the complex wiring of genes, as well as visualization of potential modulators/regulators of systems behavior under

different conditions. So far, time course gene expression studies were performed for selected individual genes only. However, such an approach neglects that there is much more information present in the expression patterns of larger sets of genes, as shown here.

Similar to the healthy chicken, we also generated a mathematical model representing the cellular components of the intestinal immune system under the *Salmonella*-disturbed condition. An additional node in this model was the number of invading *Salmonella* bacteria, in other words the number of *Salmonella* bacteria that crossed the intestinal epithelium. Furthermore, B cells were not included in this model because they are of no or less importance for clearance of *Salmonella* in chickens [12]. The mathematical model as described in Chapter 7 predicts the number of immune cells and invading bacteria as observed in real biological data rather well, suggesting that the assumed nodes and interactions are more or less correctly incorporated into the model and that we estimated the parameter values in the correct way. However, the model does not cover the dynamics of macrophages completely, which could be due to oversimplified interactions and/or incomplete relations. Also the dynamics of invading bacteria was not entirely accurately predicted by the current model, although the curve of predicted *Salmonella* bacteria reflected the biological data and also the timing of bacterial clearance. It should be emphasized that the model only represents the dynamics of the number of immune cells and invading *Salmonella* in time. Other components, such as microflora, activity of epithelial cells, cytokines, and luminal content, which also affect the adaptive capacity of the intestine to a *Salmonella* infection, were not included in the model. Nevertheless, with the current model it is, for example, possible to investigate/predict the effect of variations in the initial number of resting macrophages on the course of infection. In this case the model predicts that an initial high number of macrophages results in a decrease in the number of extracellular *Salmonella*, in other words a less severe systemic disease.

8.4 Methodology

Transcriptomics and bioinformatic analyses

At the start of the research described in this thesis (2007), the end product of microarray gene expression analyses was often a list of differently expressed genes with their accompanying nucleotide sequence and fold change in expression levels. During the course of this research many relevant bioinformatic tools and databases became available for improvement of the interpretation of microarray data. For example the Gene Ontology database [13, 14], which allows to functionally annotate (sets of) genes and to investigate whether sets of genes are enriched for

specific processes. The Database for Annotation, Visualization and Integrated Discovery (DAVID) is another example of a new bioinformatics web-based tool [15], which has integrated a variety of bioinformatic tools. For functional analysis, users can upload multiple identifiers (i.e. gene identifiers, protein identifiers, or probe identifiers) and subsequently choose their predefined background (i.e. species of interest) or upload their own custom background (i.e. all differentially expressed genes). This background is often the species of interest, but when functional annotation of the species specific genes is lacking or poor, identifiers as well as the background can be mapped to other species, for example human or mice. Although the complete genome of chicken is sequenced [16], the annotation of the chicken genome is still poor. Approximately only thirty percent of the probes on the arrays used in these studies map back to stable gene identifiers, reflecting the poor annotation of probes to genes. Therefore I searched orthologous genes within species which are closely related to chicken, however for most related species no complete genome sequences are available and also lack annotation. Therefore we used the much better annotated (nearly 100%) human or mice orthologous genes for most of the bioinformatics analyses as background. It should be noted that chicken specific processes will be lost when mapping chicken identifiers to human identifiers, and their accompanying annotation. For the analyses described in this thesis, I expect the impact of this conversion is of relative less concern because intestinal functioning and associated processes will be quite similar between mammals and birds, however there are some immunological differences.

In Chapters 2-4 we clustered genes based on their expression pattern over time. By performing guilt-by-profiling analyses and/or guilt-by-association [17] it may be possible to assign putative functions to genes that lack annotation. This is based on the assumptions that genes with similar expression patterns are involved in similar processes. However follow-up biological validation experiments are necessary, like knock-down (RNAi) or gene deletion studies, to define real gene function. Subsequently these results should be verified independently by other laboratories and then submitted to a (freely accessible) database, like Entrez Gene [18-20]. Besides a 'wet-laboratory' approach, it is also possible to perform an *in silico* validation approach, by mining different databases containing gene-gene or protein-protein interaction data [21, 22]. New developments, such as Agbase which is a curated database for functional analyses for agricultural species [23-25], will accelerate the annotation of unknown genes and the assignment of functionality to genes currently lacking annotation.

To investigate gene-gene interactions the GeneNet method [26, 27] was used, which is based on the Graphical Gaussion Models (GGM) method and which is

extremely useful for handling microarray data. We used this method because it was one of the few that could handle time-course data. Furthermore this method is computational very efficient which allows to infer a GAN with corresponding putative directions. Although this method gives putative directions to the interactions, it is difficult to distinguish between direct and indirect interactions, because the data of whole tissue was analyzed. Direct interactions can be validated by comparing the observed reaction to information that is stored in interaction databases, e.g. STRING [22]. However because our dataset contains many genes which are not mapping back to a stable gene identifier, such an analysis is in our case difficult to perform. It might be interesting to compare GANs at the network level. For example the GAN representing the *Salmonella* disturbance has a higher level of cohesiveness compared to the control GAN. In biological terms this means that the hubs of the "disturbed" GAN have more interactions with other genes than the hubs of the control GAN. Because we analyzed datasets of whole tissues, the interactions observed in our GAN represent both intracellular and intercellular interactions. An advantage of such an approach is that it represent the biology more precisely, a disadvantage is that a separation between inter- and intracellular interactions is impossible. Other advantages of this GAN approach include the possibility of using unbiased datasets, the identification of putative directions of interactions, and the identification of potential high level system regulators.

For 'simple' organisms, like bacteria and yeast, genomic information is available to find support for GANs of these organisms. For example, different gene knock-out databases exist for *Saccharomyces cerevisiae* [28]. Such databases enable the possibility to chart which genes are affected by another gene, in other words are likely to interact. For complex organisms, like chicken, such information is less-well develop. Further support for the validity of networks may be obtained from information stored in gene-gene or protein-protein interaction databases. Unfortunately, the current interaction databases contain only data on direct interaction between genes and/or proteins, whereas the presented GANs represent indirect interactions, based on mRNA expression patterns in time. Therefore, extensive biological validation of the results based on the network analysis as presented in Chapter 6 is required to sustain the proposed regulatory role of the identified hubs. For cell lines such validation can be performed by down-scaling the expression of hub genes, for example by gene knock-down technologies using RNA interference, and subsequently investigating the resulting effect on the system. For complex systems such as the chicken gut such approaches are currently impossible.

Mathematical modeling

The process of mathematical modeling can be subdivided in several stages, *i*) ask a question, *ii*) select the modeling approach, *iii*) formulate the model, *iv*) solve the model (validate if possible) and, *v*) answer the question or refine the question. When all five stages are completed and the question still remains unanswered, the process starts over again. Often stages *i* and *ii* remain unchanged, and the process is resumed from stage *iii* onwards. This can go on for multiple rounds, until the question is answered proficiently. During these iterative cycles, often new insights are gained that can lead to the formulation of new hypotheses. These new hypotheses can be used as input for further experiments and subsequent improvements of the mathematical model. When we developed the mathematical model, it was necessary to monitor the balance between too complex and too simple models. Too complex models cannot be analyzed and solved, but on the other hand too simple models will have a low predictive value.

Mathematical models are a combination of equations and variables, that describe a certain system. With such mathematical models simulations in time can be performed and predictions can be made by extrapolating the data. The variables can represent a diverse sets of values, including real numbers, integer numbers, Boolean values, or strings. The relationship between the variables is described in equations and thus represent the properties of the system. Different groups of models can be distinguished: dynamic versus static; deterministic versus stochastic (probabilistic); and linear versus non-linear models. In dynamic models the element time is taken into account, contrary to static models. In deterministic models the outcome is precisely determined by the known relations among states and events, in these models there is no randomness present. On the other hand, stochastic models do incorporate randomness, variables are not unique but determined by probability distributions. To distinguish linear from non-linear models is context dependent, but in general when all operators exhibit linearity, the model is defined as linear. Different models will answer different questions, all these models and combinations are in use and operate on different scales [29-36]. The mathematical model described in Chapter 7 is a linear deterministic dynamic model. An advantage of deterministic modeling is that it is computationally less expensive. A disadvantage is that random fluctuations that might affect reaction dynamics cannot be accounted for. We have chosen a dynamic model because these models feature more detailed and realistic system characteristics in time, rather than the representation of the system at a particular time-point. It should be realized that we only took the first steps in mathematical modeling of a very complex system.

Nevertheless, I anticipate that mathematical modeling is a powerful method to address the complexity of the intestine as a system. In this respect it is, however, important to find the right balance between complexity and predictability of the model.

8.5 Future directions and application

-omics data and intestinal functioning

In this thesis transcriptomics data were used to reflect the dynamics of the intestine during early development. In order to gain more information about processes in the gut, more transcriptomic data should be generated for different chicken lines at different locations along the intestinal tract. This will enable the identification of both line-specific and generic processes along the epithelial lining of the gut. Disturbances of gut homeostasis by different pathogens and subsequent monitoring changes in gene expression will pinpoint to different genes and processes contributing to host adaptation and defense mechanisms. In a similar way the effects of feed ingredients and feed supplements can be assessed. Ultimately, such studies will provide a comprehensive map of host encoded genes and processes that drive the behavior of the chicken intestine under a variety of conditions. Such maps provide valuable information for the search of internal and external factors that modulate the health and functioning of the intestinal tract. Further improvements of these maps may be obtained by performing meta-analyses of transcriptomic data. For human [37, 38] and mice [39, 40] data sets this meta-analyses on intestinal health has already been described. Based on the results of the latter studies, I expect that future meta-analyses of data of intestinal studies in chicken will also contribute to a better understanding of intestinal development and adaptation mechanisms. Future developments in gene expression profiling will arrive from the recent developments in the area of Next Generation Sequencing. This technique paves the way for powerful approaches like 'RNA-Seq', the successor to microarray gene expression and tiling expression arrays. With RNA-Seq more accurate mapping and quantifying of transcripts in biological samples is possible, as well as detection of post-transcriptional mutations, tissue-specific alternative splicing, and the identification of novel transcripts [41-43].

The functioning and behavior of biological systems is not only determined at the transcriptional level, but also on the level of translation (proteins) and cellular metabolism (metabolites). Therefore, the availability of proteomics and metabolomics data of gut intestinal tissue and integrated analysis of these data may further contribute to our understanding of the biology of the gut. Proteomics

focuses on the large scale quantitative profiling of proteins and investigates the composition, structure, and function of proteins in a biological sample. Proteomic studies provide additional information to transcriptomics based knowledge because the level of mRNA expression is only a rough estimate of the amount of protein, proteins may be modified by post-translational modifications that affect their activity, alternative splicing of a single transcript can give rise to multiple proteins, and proteins may form complexes with new functionalities. Additionally, metabolomic studies provide more detailed information on the physiology of cells, tissues, or organs because it profiles small-molecule metabolites, including metabolic intermediates, hormones and other signaling molecules, and secondary metabolites. For the quantitative analysis of molecular phenotypes, metabolomics is regarded as a vital element of post genomic techniques [44, 45]. Several metabolomics based databases are currently in development, for example the Human Metabolome Database (HMDB) which contains a collection of small molecule metabolites found in the human body [46]. Such databases can be used for biomarker discovery but also for validation of knowledge and models generated on the basis of transcriptomic and/or proteomic data.

By describing the (molecular) processes that are associated with two or more contrasting physiological conditions of the gut as measured at different biological levels, e.g. transcripts, proteins, metabolites, cellular dynamics, composition and activity of microbiota, etc., a more comprehensive view of the intestine as a system may be obtained. For now the major challenge is to find suitable quantitative data for these different biological levels and to connect these levels to each other. Thus the challenge for future Systems Biology approaches is to integrate all these different -omics data, in order to get a (more) complete picture of intestinal functioning and health. Different approaches can be envisaged, for example by integrating the effects of different signaling pathways, like the NFκB and PPAR signaling pathways on immune development and immune responsiveness [47], by generating genome-wide networks by massive parallel computing [48], or by integration of genome-wide transcriptomics and proteomics data [49]. Ultimately, the relationship between mRNAs, proteins, metabolites, cells, and other system components with the phenotype of the system needs to be established. Knowing these relationships is crucial for the identification of targets and factors for the controlled modulation of intestinal processes.

Improving the mathematical model

Although the mathematical model described in this thesis performs decent, further improvements are certainly required in order to cover other immunogenic and non-immunogenic aspects of gut functioning. Below I will suggest several approaches for expanding and improving the mathematical model in order to increase its predictive value.

Cytokines. For the sake of simplicity the influence of epithelial cells on model output was neglected. However, it is known that epithelial cells are able to contribute to the clearance of *Salmonella* by secretion of cytokines and the subsequent mobilization of immune cells. For example, the recruitment and activation of macrophages is influenced by the local concentrations of IL8 [50], IFNG, IL4, and IL13 [51]. Thus the model can be calibrated by the incorporation of cytokine nodes and their relationships with other nodes. For quantification of these cytokines it is possible to use the temporal quantitative gene expression measurements as described in Chapters 2 and 3.

Hubs. From the studies described in Chapter 6 we concluded that external stimuli have an effect on hub genes that potentially drive the behavior of a system. Therefore, extending the model with nodes representing these high level regulators may provide the opportunity to predict system outputs in response to external inputs. Therefore hub gens are good candidates for extending and improving mechanistic mathematical models. Each hub in the model should be represented by a separate state variable with corresponding kinetics.

Single Nucleotide Polymorphisms (*SNPs*). As shown in Chapter 5 chicken lines differ in their susceptibility to *Salmonella* and/or in the underlying phenotypes. Genetic association studies have already identified so-called quantitative trait loci (QTL) [52] involved in determining the susceptibility of chicken for *Salmonella*. Knowledge of the causal SNPs and the affected gene functions may allow to extend the model with parameters of genetic diversity and its effect on system outputs.

Applications

In the research described in this thesis, we developed basic knowledge on (molecular) processes that are associated with different physiological conditions of intestinal tissue in growing chicks. This provided information on mechanism used by the intestine as a system to adapt to physiological changes. In addition, we used modeling approaches to obtain better global views of the behavior of a complex biological system. Future progress in this area can be used to formulate new hypotheses about the behavior of (aspects of) the gut, the discovery of new biological mechanisms, and ultimately the development of tools and rational

strategies to improve intestinal health and functionality via diet and/or the host genotype. Such developments are urgently required to diminish the incidence and impact of intestinal diseases in farm animals, including chicken, and to reduce the use of antibiotics in animal production.

Diet. Various studies have shown effects of diet composition and feeding on the expression level of genes in intestinal tissue of farm animal species. Examples are the use of linseed and linseed meal [53] and inclusion of lactoferrine [54] and inuline [55] in feeds for pigs. Another example is the use of yeast cell walls, which improves the performance of broilers and has beneficial effects on gut health [56]. In these and other studies dietary induced changes in gene expression were related to immune function, gut cell proliferation and differentiation, reduction of oxidative stress, apoptosis (programmed cell death), energy metabolism (carbohydrate and fat metabolism), cell migration, gut integrity and permeability, nutrient transport, protein synthesis and mucin synthesis. Improved fundamental insight into the physiological meaning of the observed effects of specific dietary constituents and on the consequences for the functioning of the gut as a whole, will accelerate the development of rational strategies to control and modulate intestinal health and functionality via the diet. The knowledge generated in this thesis is of help for the identification of potential target for diet constituents, since we have identified key genes and key processes involved in morphological, functional, and immunological development of the chicken intestinal epithelium.

G*enotype*. The recent increase in genomic information resources for animals and the accompanying improvements in the methods for analysis and interpretation of genomic data, enables studying the intimate host-microbe-feed interactions in much more detail than ever before. In current genetic research, detailed genomic information is used to investigate correlations between genotypes (60,000-700,000 SNPs) and phenotypic traits using genome wide association studies. These genomics based approaches are already applied in advanced breeding schemes for cattle (genomic selection). Similar approaches may be applied to (aspects of) intestinal health and functionality in chicken and other farm animal species, especially when key indicator traits to quantify the health and functionality of the gastro-intestinal tract can be identified and measured at low costs in a large number of animals. The knowledge generated in this thesis is supportive in the identification of these key traits or sets of molecular components (biomarkers), since we have identified key genes and key processes that potentially drive the adaptive response of intestinal tissue in young chicks.

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Summary

To fully exploit the biological potential of the gastro-intestinal tract of farm animal species, the mechanism and regulation of processes that drive the health and functionality of the intestine need to be unraveled. To this end we focused in this thesis on 'chicken intestinal development in health and disease' by using transcriptomic and modeling approaches. In the presented research, we have set first steps in the use of Systems Biology approaches towards the identification of key components and processes involved in intestinal health and functioning.

Chapter 1 introduces the problem of the expected increased demand for animal derived food products, due to the world's increasing population and the changes in diet preferences and lifestyle. To achieve a sustainable increase of animal-based food products a more efficient primary food production system is required. By addressing processes in the intestine this may help developing more efficient production systems while concurrently taking into account both animal health and sustainability issues. Processes occurring in the intestine include; digestion; fermentation; nutrient absorption; immune recognition; immune regulation; as well as development of immune tolerance. These processes are affected by many different factors, for example the resident microorganisms, and the genetic background of the animal. To provide new tools to boost a sustainable animal production by improving gut health such as selective breeding, customizing nutrition and active intestinal health management, a better understanding of the functioning of the gut epithelium is necessary.

Chapter 2 described a functional genomics approach, using genome-wide gene expression measurements in the developing chicken intestine, to get a global view on the major biological processes in the gut and the major genes involved. Time series gene expression studies were performed in 1-21 day-old chickens on intestinal tissue. Different gene expression patterns were identified and nine gene clusters were defined. Subsequently genes residing in each cluster were functionally annotated. Genes involved in morphological and functional development were highly expressed immediately after hatch with declining gene expression afterwards. The genes involved in immunological development were divided over various gene expression clusters and based on this three distinct immunological processes could be identified over time: first innate response and invasion of immune cells; secondly immunological differentiation and specialization; and thirdly immune maturation and immune regulation. The interpretation of the gene expression data are in agreement with more traditional immunological, morphological, and functional measurements in developing chickens. Though the transcriptomic approach provided more detailed information on the biological processes.

In Chapter 3 the focus is on the developing chicken intestine disturbed by a *Salmonella* infection, and applying the same approach as described in Chapter 2. Here also temporal gene expression profiles were identified and nine gene expression clusters were defined. Subsequently the genes residing in each cluster were functionally annotated. In all nine clusters functional and morphological developmental processes dominated, but the *Salmonella* infection caused delays in several intestinal morphological processes. Metabolic processes occurred in a similar temporal frame compared to normal jejunal development. Genes involved in cell turn-over were higher expressed in *Salmonella* infected chickens compared to control chickens. Surprisingly, no clustering was found of immune related genes with comparable expression profiles. Subsequently an in-depth analysis of the immune related genes scattered over the various clusters was performed. As expected, genes involved in immunological processes were induced immediately after infection, although these processes did not dominate the global response of the intestinal tissue. The functional annotation of these genes was in agreement with measurements on different immune cell in the same tissue samples. Due to the disturbance by *Salmonella*, the well-organized temporal development of morphological processes was found to be delayed, a number of immune related genes were expressed earlier in time, whereas metabolic functional processes were almost not affected.

In chapter 4 the correlation of the number of invading pathogens with the average intestinal gene expression profiles of the expression clusters defined in Chapters 2 and 3 was investigated. Functional analyses were performed for both positive and negative correlated genes at early time-points. Moreover the top 5 ranking functional groups were analyzed in further detail. Negatively clustered genes were mainly involved in higher cell turnover and tissue repair processes which are correlated with improved adaptations of the gut and result in reduced severity of systemic disease. Contrary positively correlated genes are involved in immune related processes and wound healing, however these processes are probably a direct effect of the damage caused by the *Salmonella* infection and important for the clearance of Salmonella and repair of epithelial tissue.

Differences in susceptibility to *Salmonella* between three commercial chicken lines are described in Chapter 5. line A appeared to be the most susceptible line based on the severity of the systemic spread of *Salmonella*. Similar observations were found for the numbers of *Salmonella* in the caecum. Transcriptome analysis of intestinal tissues in lines A, B and C on the first 2 days post infection showed a much stronger transcriptional response in line A compared to lines B and C. The affected genes of line A are involved in cell cycle functions, metabolic activity and

immunological related processes. Differences between the lines in weight gain and the number of immune cells in the intestinal tissues could also be observed. We concluded that that genetically different chicken employ different routes and processes to counteract an infection with *Salmonella*. We also conclude that the difference between lines A compared to lines B and C may be related to differences in in the maturity and or integrity of the epithelial cell layer.

In Chapter 6 we inferred gene association networks (GANs) from time-course gene expression data. They show that gene associations exist that deviate from associations seen in cluster and functional and/or pathway analyses. By calculating network statistics we show that the GANs follow a power law, i.e. scale-free network, which is observed for many biological networks. The inferred GANs provide a global picture of highly regulated processes in a complex tissue under different environmental conditions. We observed a shift in the topology and functionality of these networks when comparing *Salmonella* infected to healthy chickens. By calculating the putative direction of the gene-gene interactions in the GAN, we found that highly connected genes (hubs) influence their direct neighbors over time and not vice versa. The set of hub genes of the control GAN completely differed from the hubs identified in the intestinal tissue of infected chicken. In the infected chickens hubs were found to be associated to cellular communication as well as cytoplasmic regulation, whereas in the control chickens hubs were mainly involved in transcriptional regulation. A number of the cell-cell communication hubs are involved in host defense and pathogen response functions, whereas the control hubs are more associated to developmental processes.

In Chapter 7 we describe the development of a mathematical model representing the development and responsiveness of the cellular compartment of the chicken intestinal immune system. To model the immune response in time, different ordinary equations were generated for each of the selected state variables. We found a good correlation between the model output and the real biological data used for parameterization. In addition the model output fitted with experimental data of early immune intestinal development of three different chicken lines. We calculated R-square values which indicated that we determined both the selected cell types (nodes) and the assumed interactions (edges) more or less correctly. The model predicted the dynamics of intestinal immune cells after an infections with *Salmonella* well. Also the curve of predicted *Salmonella* bacteria reflected the biological data as well as the timing of bacterial clearance. By parameterizing the variables with a genetically different chicken line resulted in similar output for the number of *Salmonella* as observed in the accompanying bacteriological data.

In Chapter 8 the major findings of this thesis are discussed in a broader context. It includes a discussion on the biological processes and high level regulators that potentially drive the development and adaptation of the gut-system under normal conditions and under *Salmonella* disturbed conditions and how this information contributes to our understanding of the mechanism used by intestinal tissue to adapt to environmental changes. Also the different methods used for the research described in this thesis are discussed. Further attention is paid to current challenges related to network validation, the advantages and disadvantages of mathematical modeling and how to improve the predictability of the mathematical model. Finally, new research ideas regarding the use of -omics data in relation to intestinal functioning and health are discussed as well as potential application of the knowledge generated in this research.

Samenvatting

Darmgezondheid is een belangrijke voorwaarde voor een duurzame dierlijke productie. Omdat er aanzienlijke variatie is in darmgezondheid en functionaliteit tussen dieren, is er ook veel te winnen in dit opzicht. Om het biologische potentieel van het maag-darmkanaal van dieren ten volle te kunnen benutten, moet eerst meer inzicht vergaard worden over de mechanismen en de regulatie van de belangrijkste darmprocessen. Met het onderzoek beschreven in dit proefschrift is basiskennis ontwikkeld over de (moleculaire) processen van darmweefsel en zijn deze gekoppeld aan verschillende fysiologische omstandigheden van het darmweefsel. Om de processen en de verschillen in processen in beeld te brengen hebben we gebruik gemaakt van een transcriptoom (verzameling van RNA-moleculen die betrokken zijn bij transcriptie) en modelmatige benadering. Ook hebben we in dit onderzoek de eerste stappen gezet op het gebied van de systeem biologie. Hiermee proberen we belangrijke componenten en processen die betrokken zijn bij darmgezondheid en darmfunctionaliteit te identificeren en hun onderlinge relaties vast te stellen.

Hoofdstuk 1 schetst het scenario dat de verwachte grotere vraag naar voedingsproducten van dierlijke oorsprong als gevolg van de groeiende wereldbevolking en de wereldwijde veranderingen in dieetvoorkeuren en levensstijlen een probleem kunnen worden. Om op een duurzame manier aan de grotere vraag te kunnen voldoen zijn efficiëntere primaire voedselproductie-systemen nodig. Meer kennis over de verschillende processen in de darm kan bijdragen aan de ontwikkeling van zulke efficiëntere productiesystemen. Tevens kan deze kennis bijdragen aan een verbetering van de (darm)gezondheid van productiedieren en aan een reductie van het gebruik van antibiotica. De belangrijkste processen van de darm zijn spijsvertering, fermentatie, opname van voedingsstoffen, immuun herkenning, immuun regulatie en de ontwikkeling van immuun tolerantie. Al deze processen worden beïnvloed door verschillende factoren zoals de darmflora, de diervoeding en de genetische achtergrond van het dier. Een beter begrip over de werking van de darm kan ook leiden tot nieuwe toepassingen in de veehouderij om via een verbeterde darmgezondheid de dierlijke productie op een duurzame wijze te verhogen. Hierbij kan gedacht worden aan het aanpassen en optimaliseren van diervoeding, het fokken op darmkenmerken of het ontwikkelen van een actief darmgezondheid management systeem.

Hoofdstuk 2 beschrijft een functionele genomica aanpak om een breed overzicht te krijgen van de belangrijkste biologische processen in een ontwikkelende darm en de genen die daarbij betrokken zijn. Hierbij wordt gebruik gemaakt van opeenvolgende genoom-brede genexpressie metingen in de zich ontwikkelende darm van kuikens van 1 tot 21 dagen oud. Verschillende genexpressie patronen

werden geïdentificeerd en op basis van die expressiepatronen werden negen gen-clusters gedefinieerd. Vervolgens zijn de genen van elk cluster functioneel geannoteerd (het voorzien van biologische informatie en eigenschappen van genen). Hierbij werd gevonden dat genen die betrokken zijn bij morfologische (opbouw van de darmstructuur) en functionele ontwikkelingen een hoge expressie hadden direct na geboorte en daarna een afnemende expressie. De genen die betrokken zijn bij immunologische ontwikkelingen waren verdeeld over de verschillende genexpressie clusters. Door een gedetailleerde analyse van alle immuun gerelateerde genen konden drie verschillende immunologische processen in de tijd geïdentificeerd worden: 1) 'innate immuunrespons' en de 'invasie van immuuncellen'; 2) 'immunologische differentiatie en specialisatie'; en 3) 'ontwikkeling van het immuunsysteem' en 'immuun regulatie'. De interpretatie van de genexpressie gegevens zijn in overeenstemming met de bevindingen van meer traditionele immunologische, morfologische en functionele metingen in opgroeiende kuikens. De transcriptoom aanpak geeft meer gedetailleerde informatie over de verschillende biologische processen.

In hoofdstuk 3 is eenzelfde benadering als in hoofdstuk 2 toegepast voor het bestuderen van de processen in een ontwikkelende kippendarm die verstoord is door een infectie met *Salmonella.* Ook hier zijn temporele genexpressie profielen geïdentificeerd, negen gen-clusters gedefinieerd en de genen van elk cluster functioneel geannoteerd. In alle negen genclusters domineerden de functionele en morfologische ontwikkelingsprocessen, echter de *Salmonella* infectie veroorzaakte vertragingen in verschillende morfologische processen. Tussen de gezonde- en *Salmonella*-geïnfecteerde dieren werden geen grote verschillen gevonden in (de timing) van metabole processen. Genen die een rol spelen bij de turnover van cellen kwamen hoger tot expressie in *Salmonella* geïnfecteerde dieren dan in de controle kippen. Verrassend genoeg vonden we geen opvallende clustering van immuun gerelateerde genen. Wel zagen we dat de expressie van een aantal immuun gerelateerde genen onmiddellijk na de infectie met *Salmonella* geïnduceerd werden. Het expressiepatroon van de immuun gerelateerde genen en hun functionele annotatie kwam goed overeen met de metingen die gedaan werden aan verschillende typen immuuncellen in dezelfde weefselmonsters. We concluderen in dit hoofdstuk dat als gevolg van de verstoring door *Salmonella*, de goed georganiseerde temporele ontwikkeling van morfologische processen wordt uitgesteld, dat een aantal immuun gerelateerde genen eerder in de tijd tot expressie komt, en dat metabole processen bijna niet aangetast worden.

In hoofdstuk 4 is de correlatie beschreven tussen het aantal binnendringende ziekteverwekkers (*Salmonella*) en de (gemiddelde) genexpressie profielen zoals

gevonden in de hoofdstukken 2 en 3. Functionele analyses zijn uitgevoerd voor zowel positief als negatief gecorreleerde genen gedurende de vroege fase van infectie. Negatief gecorreleerde genen zijn vooral betrokken bij een hogere cel turnover en weefselherstel. Deze genen zijn dus betrokken bij een verbeterde aanpassing van de darm en dit resulteert waarschijnlijk in een afname van de ernst van de ziekte. Daarentegen zijn positief gecorreleerd genen vooral betrokken bij immuun gerelateerde processen en bij 'wondgenezing'. Deze processen zijn waarschijnlijk een direct effect van de schade veroorzaakt door de *Salmonella* infectie en zijn van belang voor het afdoden van *Salmonella* bacteriën en het herstel van beschadigd darmweefsel.

In hoofdstuk 5 zijn drie commerciële kippenlijnen bestudeerd die verschillen in hun gevoeligheid voor *Salmonella.* Lijn A bleek de meest gevoelige lijn te zijn op basis van de systemische verspreiding van *Salmonella* en het aantal *Salmonella* in het cecum. Transcriptoom analyses van darmweefsel van lijn A, B en C op de eerste twee dagen na infectie liet zien dat lijn A een veel sterkere transcriptionele respons heeft in vergelijking met lijnen B en C. Deze genen zijn betrokken bij celcyclus, metabole activiteit en immunologische processen. Ook zijn er verschillen tussen de lijnen waargenomen met betrekking tot hun gewichtstoename en het aantal immuuncellen in het darmweefsel. Aan de hand van deze data konden we vaststellen dat dieren met een verschillende genetische achtergrond verschillende processen toepassen om te reageren op een infectie met *Salmonella*. Het verschil tussen lijn A met lijnen B en C kan mogelijk worden gerelateerd aan verschillen in de volgroeiing en/of de integriteit van de darmwand op de dag van het uitkomen van het ei.

In hoofdstuk 6 hebben we gen-associatie netwerken (GANs) van temporele genexpressie data afgeleid. Deze GANs tonen aan dat er associaties tussen genen bestaan die anders zijn dan de associaties die te zien zijn in clusteranalyses en functionele en/of pathway analyses. Door het berekenen van netwerk statistieken is te zien dat de GANs een 'power-law' volgen en 'scale-free' zijn, hetgeen een aanwijzing is dat ze biologische betekenis hebben. De GANs geven een totaalplaatje van de gereguleerde processen in een complex stukje darmweefsel onder verschillende omstandigheden. Bij de vergelijking tussen gezonde en *Salmonella* geïnfecteerde kuikens zagen we een verschuiving optreden in de topologie en de functionaliteit van de GANs. Door te kijken naar de richting van de gen-gen interacties in de GANs hebben we (hub) genen gevonden die veel interacties hebben met andere genen en die de expressie van veel andere genen beïnvloeden. De set van hubs van de controle GAN verschilt volledig van die van het darmweefsel van geïnfecteerde kuikens. De hubs van de geïnfecteerde kuikens

hebben vooral te maken met intercellulaire communicatie en cytoplasmatische regulatie. Een aantal hubs van geïnfecteerde kuikens zijn ook betrokken bij afweer en bij pathogeen respons functies. De hubs van de controlegroep zijn vooral betrokken bij de transcriptionele regulatie van cellulaire ontwikkelingsprocessen.
In hoofdstuk 7 beschrijven we een wiskundig model dat de ontwikkeling en het reactievermogen vertegenwoordigt van de cellulaire tak van het immuunsysteem in de kippendarm. Om de ontwikkeling en de immuunrespons in de tijd te modelleren zijn verschillende differentiaal vergelijkingen gegenereerd voor elk van de geselecteerde variabelen. We vonden een goede correlatie tussen de uitkomsten van het model en de biologische gegevens die gebruikt werden voor de parameterisatie van het model. Bovendien voorspelt het model het verloop van experimentele gegevens, waarbij naar de vroege ontwikkeling van het immuun systeem in de darm werd gekeken, bij drie verschillende kippenlijnen. Op basis van de R-kwadraat waarden bleek dat we de geselecteerde celtypen en de veronderstelde interacties correct bepaald hadden. Het model voorspelde de dynamiek van de immuuncellen in de darm na een infectie met *Salmonella* goed. Ook de curve van het voorspelde aantal bacteriën klopte met de biologische gegevens, evenals het tijdstip wanneer de kuikens weer volledig vrij zijn van *Salmonella*. Door de modelvariabelen een andere parameterwaarde te geven konden we een simulatie maken die overeenkomt met bacteriologische data van kippenlijnen met een verschillende genetische achtergrond.
In hoofdstuk 8 worden de belangrijkste bevindingen van dit proefschrift besproken in een bredere context. Het omvat een discussie over de biologische processen en over 'high-level' regulatoren die mogelijk de ontwikkeling en aanpassing van het darm-systeem aansturen in een niet-verstoorde situatie en onder een *Salmonella* verstoorde situatie. Verder wordt bediscussieerd hoe deze informatie bijdraagt aan onze kennis over de mechanismen die gepaard gaan met aanpassingen van de darm aan veranderende omstandigheden. vervolgens worden de verschillende methoden die gebruikt zijn bij dit onderzoek bediscussieerd. Daarna worden een aantal toekomstige uitdagingen beschreven die ons te wachten staan voor de validatie van de uitkomsten van onderzoek aan gen-netwerken. Ook worden de voor- en nadelen van wiskundig modelleren worden besproken, hoe het model gebruikt kan worden en hoe de voorspelbaarheid van het wiskundige model verbeterd zou kunnen worden. Tenslotte worden er nieuwe ideeën voor onderzoek over het gebruik van -omics gegevens voor een beter begrip van darmwerking en darmgezondheid besproken en wordt gerefereerd aan mogelijke toepassing van de kennis uit dit onderzoek.

Curriculum Vitae

On January 29th of 1984 Dirkjan Schokker was born in Dronten, and he grew up in Ermelo. His high school diploma was obtained in 2002 at the school 'Christelijk College Groevenbeek' in Ermelo. In that same year he started his Bachelor study Biology at Utrecht University and completed this in the year 2005. Immediately after obtaining his BSc, he started the Master 'Developmental Biology and Biomedical Genetics' which was also taught at Utrecht University. Here he followed two internships, the first internship (6 months) he investigated root development in plants, about new (unknown) genes and their promoter sequences which are involved in root growth. In his second internship (9 months), he has conducted meta-analysis of linkage and gene expression data from several human autoimmune diseases, to identify common regulatory mechanism underlying these diseases. His thesis was a literature review entitled 'Immune receptor diversity in differential species', the function of immune receptors was investigated in different species by the use of several bioinformatic tools. After his degree he began his work as a PhD at Wageningen UR in the group of Animal Breeding and Genetics Centre (ABGC).

Op 29 januari 1984 werd Dirkjan Schokker geboren te Dronten, en is opgegroeid in Ermelo. Zijn VWO diploma heeft hij in 2002 behaald op het Christelijk College Groevenbeek te Ermelo. In dit zelfde jaar is hij met de bachelor studie Biologie aan de Universiteit Utrecht begonnen en heeft deze afgerond in 2005. Direct daarna is hij begonnen met de master 'Developmental Biology and Biomedical Genetics' aan de Universiteit Utrecht. Tijdens zijn master heeft hij twee stages gevolgd, in de eerste stage (6 maanden) was het onderwerp wortelontwikkeling bij planten, waarbij is gekeken naar nieuwe (onbekende) genen en hun promotor sequenties die betrokken zijn bij wortelgroei. In zijn tweede stage (9 maanden), heeft hij meta-analyses uitgevoerd op 'linkage' en gen expressie data van verscheidene humane auto-immuun ziektes, om algemene mechanismen te identificeren die ten grondslag liggen aan deze ziektes. Naast deze stages heeft hij zijn master thesis, een literatuur onderzoek gedaan over 'Immune receptor diversity in different species', waarbij door gebruik te maken van meerdere bioinformatische tools de functie van immuunreceptoren was onderzocht in verschillende diersoorten. Na het behalen van zijn master diploma is hij gaan werken bij Wageningen UR als Assistent In Opleiding in de vakgroep 'Animal Breeding and Genetics Centre' (ABGC).

List of publications

Schokker, D., Peters, T.H.F., Hoekman, A.J.W., Smits, M.A., Rebel, J.M.J, **Differences in the early response of hatchlings of different chicken breeding lines to Salmonella infection**, Poultry Science 91 :346–353 (2012)

Book: **Systems Biology and Livestock Science**, chapter 10 together with M.A. Smits. ISBN: 978-0-8138-1174-1 (2011)

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Dirkjan

Training and Supervision Plan

The basic package

WIAS Introduction Course	2008
Course on philosophy of science and/or ethics	2010

Scientific exposure

Animal Disease genomics: Opportunities and Applications, Edinburgh	2008
3rd Symposium: Mucosal Immunology, Rotterdam	2008
EADGENE: Genomics for Animal Health: Outlook for the Future, Paris	2009
Animal Genomics and Animal Health, Paris	2010
International Conference on Systems Biology, Edinburgh	2010
Fundamental Physiology and Perinatal Development in Poultry, Wageningen	2011

Seminars and workshops

Integrative Bioinformatics: At the cutting edge of network analysis and biological data integration, Amsterdam	2007
Control Theory for Systems Biology, Groningen	2007
WIAS Science Day, Wageningen	2008
Post-analyses Workshop, Lelystad	2008
WIAS Science Day, Wageningen	2009
Systems Genetics: From man to microbe from genotype to phenotype, Groningen	2009
Systems Biology Day IP/OP projects WUR, Wageningen	2010
GENESYS, Edinburgh	2010
WIAS Science Day, Wageningen	2011
Systems Biology Day IP/OP projects WUR, Wageningen	2011

In-depth studies

Statistics for Life Sciences	2008
Mathematical modelling in biology	2008
Advanced visualisation, integration and biological interpretation of -omics data	2009
Eykman Graduate School – Immune	2010

Professional skills support courses

Course Techniques for Scientific Writing	2008
Project and Time management	2008
PhD Competence assessment	2008
Career Orientation	2011
Career Perspectives	2011

Research skills training

Preparing own PhD research proposal	2007

Management skill training

Organisation of seminars and courses; GeneSpring GX Course	2008
WIAS Associated PhD Students-Council	2010/2011

Education and training total **42 ECTS**

Colophon

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