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Immunomodulatory effects of MicroRNAs and fructooligosaccharide during *Salmonella* Enteritidis Infection in young chickens

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Résumé

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Résumé:

À l'échelle internationale, les toxi-infections d'origine alimentaire font l'objet d'une attention croissante, dont la salmonellose qui est une toxi-infection très répandue dans le monde. Elle représente environ 93,8 millions de cas et est la cause de 155 000 décès par an. *Salmonella* Enteritidis (SE), la bactérie causant la salmonellose, provoque non seulement des pertes économiques énormes pour le secteur de la volaille, mais nuit aussi gravement à la santé publique humaine. Les volailles sont considérées comme une source importante pour le portage de SE et une source majeure de la salmonellose chez l'homme. SE a également tendance à être très résistante aux substances antimicrobiennes, ce qui complique le traitement des maladies à la fois chez les animaux et l'homme. Une meilleure compréhension de la résistance immunologique de l'hôte et des mécanismes de réponse chez les volailles devrait être une priorité absolue. Le but de la recherche décrite dans cette thèse a été divisé en deux parties: (1) identifier les micro-ARN (miARN) spléniques et les ARNm qui ont été différentiellement exprimés après l'infection des poulets avec SE en utilisant le séquençage de l'ARN; (2) examiner si l'addition de fructo-oligosaccharides (FOS) modifie l'expression des gènes inflammatoires impliqués dans la signalisation dépendante MyD88 dans les tissus immunitaires au cours de l'infection à *Salmonella*. Pour atteindre ces objectifs, la stratégie de la recherche impliquait la construction d'un modèle amélioré de la maladie, l'analyse transcriptonique, l'identification des gènes et des voies de résistance, et l'évaluation de l'effet de l'addition de FOS, à la fois par la voie alimentaire et lors de la culture *in vitro* de cellules immunitaires des volailles.

Dans la première partie, des micro-ARN et des transcrits de gènes (ARNm) différentiellement exprimés, ainsi que les voies de signalisation ont été étudiés chez des volailles qualifiées de résistantes (R, les volailles chalengées avec SE présentaient des symptômes cliniques légers et moins de 10^5 cfu SE / 10 µL de sang), de sensibles (S, les volailles chalengées avec SE présentaient des symptômes cliniques sévères et plus de 10^7 cfu SE / 10 µL de sang) par rapport à des volailles contrôles (C, non-challengées et pas de SE dans le sang) en utilisant le miARN splénique et le transcriptome. Au total, 934 gènes significativement exprimés différentiellement (DE) et 32 DE miARN ont été identifiés lors des comparaisons entre les oiseaux C, R et S. Rapporté pour la première fois dans ce travail, les DE gènes impliqués dans la voie de signalisation Forkhead box O (FoxO), en particulier FoxO3, ont été identifiés comme des marqueurs potentiels de la résistance de l'hôte à l'infection par SE. Il y a des preuves de l'interférence entre ces voies, contribuant peut-être à la susceptibilité à l'infection à Salmonella, y compris la voie de signalisation FoxO, l'interaction des récepteurs de cytokine-cytokine et la voie de

signalisation Jak-STAT. Fait important, la signalisation du TLR4 a également été significativement enrichie chez les oiseaux C, R et S. De plus, deux DE miARN, gga-miR-101-3p et gga-miR-155, ont directement réprimé l'activité du gène rapporteur de la luciférase en se liant aux régions 3' non traduites des gènes cibles immunitaires *IRF4* et *LRRC59*, respectivement. Lorsque gga-miR-155 et l'interférence gga-miR-101-3p étaient surexprimés dans les macrophages HD11 de poulet, l'expression de leurs gènes cibles était significativement modifiée. Ces deux miARN ont été identifiés comme étant potentiellement associés à l'infection à SE.

La deuxième partie de l'étude a examiné si la fourniture de FOS peut modifier l'expression des gènes inflammatoires impliqués dans la signalisation MyD88-dépendante au cours de l'infection à Salmonella. Il a d'abord été nécessaire de déterminer le niveau optimal d'ajout de FOS dans l'alimentation pour une protection efficace contre l'infection à *Salmonella*. Des poussins d'un jour exempts de pathogènes spécifiques (SPF) ont été nourris avec un régime de base contenant 0, 10, 20 et 30 g de FOS / kg. Trois jours après l'éclosion, tous les poussins ont été challengés par voie orale avec SE. Les concentrations les plus faibles de FOS ont réduit la charge bactérienne dans le foie et le caecum, diminué les taux sériques d'interleukine-1β et diminué l'abondance relative des transcrits du TNF-α et de l'IL-6 dans les amygdales spléniques et caecales. Ces résultats suggèrent un effet bénéfique chez les jeunes poussins de l'ajout de FOS, probablement à la dose de 1% du régime alimentaire, à titre prophylactique contre l'infection par SE.

Les effets de l'addition de FOS par la voie alimentaire sur l'expression de gènes inflammatoires et de gènes de signalisation TLR4 ont également été étudiés chez de jeunes poulets et les actions directes des FOS sur des macrophages de poulets challengés avec la toxine bactérienne LPS. Trois jours après l'éclosion, des oiseaux de deux groupes de traitement (régimes avec de 1% ou sans FOS) ont également été soumis à challenge oral avec SE ou un tampon phosphate. Les FOS alimentaires ont significativement réduit l'expression des gènes des cytokines pro-inflammatoires IL-6 et TNF-α, ainsi que l'abondance des transcrits des gènes *TLR4*, *MyD88*, *TRAF6* et *NF-κB* liés à l'inflammation dans la rate et dans les amygdales caecales au cours de l'infection par SE chez les jeunes poulets. Lors de la culture *in vitro* des macrophages HD11de poulet, l'exposition aux FOS a directement augmenté l'expression de l'IL-6 et du TNF-α et a réduit l'ampleur de l'augmentation de l'abondance des facteurs pro-inflammatoires, provoquée par l'ajout de LPS. Pris ensemble, ces résultats fournissent de nouvelles informations que les FOS peuvent réduire la production des cytokines pro-inflammatoires grâce à la signalisation TLR4-MyD88-dépendante au cours des premiers stades après l'infection par Salmonella. Il est souligné que de plus amples recherches de ce rôle immunomodulateur direct de FOS sur la signalisation TLR4 sont justifiées.

En conclusion, cette recherche avec des volailles a systématiquement révélé de nouvelles informations sur le mécanisme immunitaire de l'hôte lui fournissant une certaine protection contre *Salmonella* en utilisant le séquençage à haut débit combiné avec une stratégie expérimentale améliorée. Plusieurs voies de signalisation importantes et des miARN ont été identifiés et feront l'objet de recherches futures. De plus, des preuves que les FOS ont une influence régulatrice directe sur l'immunité innée chez les poulets ont été obtenues. Ces découvertes mécanistiques aideront à

comprendre la résistance et la susceptibilité à l'infection à Salmonella dans les premières phases de la réponse immunitaire de l'hôte, fourniront de nouvelles approches pour développer des stratégies de prévention et de traitement des salmonelloses, et aideront à renforcer la résistance innée par la sélection génétique.

Mots-clés: *Salmonella* Enteritidis; miARN et ARNm; Séquençage de l'ARN; exprimé de manière différentielle; FOS; Cytokines pro-inflammatoires; voie de signalisation

Abstract

Peng LI. (2018). Immunomodulatory effects of microRNAs and fructooligosaccharide during *Salmonella* Enteritidis Infection in young chickens. (PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, Liège University, 148 p., 13 tables, 20 figures.

Summary:

Internationally, foodborne diseases are receiving increasing attention. Salmonellosis is one of the most common foodborne diseases worldwide, accounting for around 93.8 million foodborne illnesses and 155,000 deaths per year. As a foodborne disease, *Salmonella* Enteritidis (SE) not only causes huge economic losses to the poultry industry, but also seriously threatens human public health. Poultry are considered to be important sources and carriers of the disease. SE also tends to be highly resistant to multiple antimicrobials, which has the potential to complicate treatment of animal and human disease. An improved understanding of host immunological resistance and response mechanisms in chickens should be a top priority. The aim of the research described in this thesis was divided into two parts: (1) To identify the splenic microRNAs and mRNAs that were differentially expressed following infection of chickens with *Salmonella* Enteritidis using RNA sequencing; (2) To investigate whether fructooligosaccharides (FOS) addition alters the expression of inflammatory genes involved in MyD88-dependent signaling in immune tissues during *Salmonella* infection. To achieve these objectives, the research strategy involved construction of an improved disease model, transcriptome screening, identification of resistance genes and pathways, and evaluating the effect of FOS addition, both by dietary supplementation and by exposure of chicken immune cells *in vitro*.

In the first part, differentially expressed microRNAs and gene transcripts (mRNAs), as well as signaling pathways were investigated in resistant (R, SE challenged-slight clinical symptoms and $< 10^5$ cfu SE / 10 μL blood), susceptible (S, SE challenged-severe clinical symptoms and $> 10^7$ cfu SE / 10 μL blood) and control birds (C, non-challenged, no SE in blood) using the splenic microRNAome and transcriptome. A total of 934 significant differentially expressed (DE) genes and 32 DE miRNAs were identified in comparisons among the C, R and S birds. First reported here, the DEG involved in the Forkhead box O (FoxO) signaling pathway, especially FoxO3, were identified as potential markers for host resistance to SE infection. There was evidence of cross-talk among these pathways, perhaps contributing to susceptibility to *Salmonella* infection, including the FoxO signaling pathway, cytokine-cytokine receptor interaction and Jak-STAT signaling pathway. Importantly, TLR4 signaling was also significantly enriched among C, R and S birds. In addition two DE miRNAs, gga-miR-101-3p and gga-miR-155, directly repressed luciferase reporter gene activity by binding to 3'-untranslated regions of the immune-related target genes *IRF4* and *LRRC59*. When gga-miR-155 and interference gga-miR-101-3p were over-expressed in chicken HD11 macrophages, expression of their

target genes was significantly altered. These two miRNAs were identified as candidates being potentially associated with SE infection.

The second part of the study investigated whether provision of FOS altered the expression of inflammatory genes involved in MyD88-dependent signaling during *Salmonella* infection. It was first necessary to determine the optimum level of adding dietary FOS for effective protection against *Salmonella* infection. One day-old specific-pathogen-free (SPF) chicks were fed throughout with a basal diet containing 0, 10, 20, and 30 g/kg supplemental FOS. Three days post-hatch, all chicks were orally challenged with SE. Lower concentrations of dietary FOS reduced the bacterial burden in liver and cecum, decreased the serum levels of interleukin-1β, and decreased relative abundance of *TNF-α* and *IL-6* transcripts in spleen and cecal tonsils. These results suggest a beneficial effect of adding FOS, probably at 1% of the diet of young chicks, prophylactically against SE infection.

The effects of dietary FOS addition on the expression of inflammatory genes and TLR4 signaling genes were also investigated in young chickens and direct actions of FOS on chicken macrophages challenged with the bacterial toxin, LPS, were examined. Three days post-hatch, birds from two treatment groups (diets with or without 1% FOS) were also orally challenged with SE or vehicle PBS. Dietary FOS significantly reduced the gene expression of pro-inflammatory cytokines *IL-6* and *TNF-α*, as well as the transcript abundance of inflammation-related pathway genes *TLR4, MyD88, TRAF6* and *NF-κB* in spleen and in cecal tonsils during *S.* Enteritidis infection in young chickens. Using HD11 chicken macrophages *in vitro*, exposure to FOS directly increased the expression of *IL-6* and *TNF-α* and reduced the extent of increase in abundance of pro-inflammatory factors, otherwise provoked by added LPS. Taken together, these findings provide novel information that FOS may reduce production of the pro-inflammatory cytokines through TLR4-MyD88-dependent signaling during the early stages after *Salmonella* infection. It is emphasized that further research of this direct immunomodulatory role of FOS on TLR4 signaling is warranted.

In conclusion, this research with chicks has systematically exposed novel information on the immune mechanism of the host in providing some protection against *Salmonella* by use of a high-throughput sequencing combined with an improved experimental design strategy. Several important signaling pathways and miRNAs have been identified and will be the focus of future research. In addition, evidence for FOS having a direct regulatory influence on innate immunity in chickens was obtained. These mechanistic findings will help facilitate the understanding of resistance and susceptibility to *Salmonella* infection in the earliest phases of the host immune response, they will provide new approaches for developing strategies for *Salmonella* prevention and treatment, and they may aid in enhancing innate resistance using genetic selection.

Keywords: *Salmonella* Enteritidis, miRNA and mRNA, RNA sequencing, differentially expressed, FOS, pro-inflammatory cytokines.

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List of Abbreviations

C	Non-challenged
cfu	Colony forming units
CLRs	C-type lectin receptors
DCs	Dendritic cells
DE	Differentially expressed
DEG	Differentially expressed genes
DFM	Direct-fed microbials
dpi	Days post infection
EU	European Union
FC	Fold change
FDR	False discovery rate
FOS	Fructooligosaccharides
FPKM	Fragments per kilobase of exon per million
GIT	Gastrointestinal tract
GO	Gene ontology
GPR	G protein-coupled receptor
KEGG	Kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
MicroRNA	miRNA
MOS	Mannanoligosaccharides
NLRs	NOD like receptors
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PRRs	Pattern-recognition receptors
qPCR	Real-time polymerase chain reaction
R	Challenged-resistant
RISC	RNA-induced silencing complex
RNA-seq	RNA sequencing
S	Challenged-susceptible
SCFA	Short-chain fatty acid
SE	*Salmonella* Enteritidis
SPF	Specific pathogen free
Th	Helper T cell
TLR	Toll-like receptors
UK	United Kingdom
UTR	Untranslated region

1

General Introduction

Internationally, foodborne diseases are receiving increasing attention. Salmonellosis is one of the most common foodborne diseases worldwide, accounting for around 93.8 million foodborne illnesses and 155,000 deaths per year (Eng et al., 2015; Heredia and García, 2018). Reports from the United States indicate that more than one million people are sickened by *Salmonella* each year, and in approximately 20% of these cases, poultry was the pathogenic vehicle (Hoffmann et al., 2015). In the United States, data from 2000 to 2008 give an estimated average cost in health care of this foodborne illness of $55.5 to $93.2 billion (Scharff, 2015; Heredia and García, 2018). Reports from the EU in 2015 showed 94,625 confirmed cases of salmonellosis in humans and 126 deaths (EFSA-ECDC, 2016).

Salmonella Enteritidis (SE) is a Gram-negative enteric pathogen, infection with which does not cause significant disease or mortality in poultry, but birds can carry the bacteria for several weeks without presenting any clinical signs, thereby constituting an insidious risk for public health (Barrow et al., 2012; Calenge and Beaumont, 2012; Kogut and Arsenault, 2017). Although *Salmonella* contamination can be significantly reduced using control measures in poultry, there was a considerable increase in reported *Salmonella* cases in the EU (EFSA and ECDC, 2016) and UK (Inns et al., 2015). However, with recent concerns of bacterial antibiotic resistance and the presence of antibiotic residues in meat, alternative methods such as dietary interventions are being evaluated to reduce or eliminate *Salmonella* colonization in chickens (Babu and Raybourne, 2008). Notable among the interventions are the use of prebiotics and direct-fed microbials (DFM) in animal feeds as they have been shown to have immunomodulatory effects by boosting the host immune response and thus conferring resistance to infections.

The term "prebiotic" was first coined by Gibson and Roberfroid (1995). Prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria, which can improve the host health." Currently, prebiotics are more broadly defined as any type of dietary compound that has beneficial physiological effects on the host by being metabolized by intestinal microorganisms (Bindels et al., 2015), having as a consequence antimicrobial activity against pathogens (Pineiro et al., 2008). Fructooligosaccharides (FOS) are short-chain non-digestible carbohydrates extracted from plant sources (e.g., chicory root, onion, beet and cane sugar) and are considered to have a prebiotic effect in maintaining intestinal health (Meyer and Stasse-Wolthuis, 2009; Sarao and Arora, 2017), improving growth performance, and modulating immune function in animals (Flickinger et al., 2003; Shang, 2014; Tran et al., 2018). It is suggested that well-known immunomodulation mechanisms of FOS-inulin encompass stimulation of growth and activity of lactic acid bacteria, but can also be mediated by fermentation products of these bacteria i.e. short chain fatty acids (SCFA). Recent in vitro studies, however, have demonstrated that FOS-inulin can be recognized directly by intestinal epithelial cell lines (Zenhom et al., 2011; de Kivit et al., 2011) and immune cells (Babu et al. 2012; Ortega-González et al., 2014; Capitán-Cañadas et al., 2014), through inhibiting the NF-κB signaling pathway, eventually reducing production of pro-inflammatory cytokines. In addition, in TLR4-KO mice, FOS/inulin-induced secretion of monocyte chemoattractant protein

1 (MCP-1) was also reduced in colonic explants compared with the untreated controls (Capitán-Cañadas et al., 2014). Although the potential effect of FOS-inulin on regulation of the immune response has been widely addressed in humans and rats (Ferenczi et al., 2016; Ortega-González et al., 2014), little information is available in chicks.

Recently there is increasing evidence that the microRNAs (miRNAs) play important roles in regulating the innate immune response induced by bacteria (Das et al., 2016; Eulalio et al., 2012; Maudet et al., 2014; Staedel and Darfeuille, 2013). MiRNAs have been identified as key regulators of gene expression at the post-transcriptional level. These small RNAs have been demonstrated to have important functions in a variety of biological processes including the cell cycle, differentiation, apoptosis and pathogenesis (Ambros, 2004; Filipowicz et al., 2008; Krol et al., 2010; Yates et al., 2013). Previous studies have shown that miRNAs, such as *miR-146a*, *miR-155* and *Let-7* and their target gene transcripts are involved in the regulation of the immune response against *Salmonella* or lipopolysaccharide infection in mice (O'Neill et al., 2011; Eulalio et al., 2012; Schulte et al., 2011) and swine (Bao et al., 2015; Bao et al., 2014; Yao et al., 2016a; Yao, et al., 2016b). However, there is still only limited information about the possible function of miRNAs in the host response and resistance to *Salmonella* infection in chickens.

Although the miRNAs and FOS have potential immunoregulatory functions in *Salmonella* infection, a better understanding of host mechanisms of immunological resistance deserves priority attention in chickens.

1 Overview of *Salmonella* Enteritidis

Globally, *Salmonella enterica* subsp. *enterica* is one of the most commonly reported causes of foodborne human gastroenteritis, a disease characterized by gut inflammation and self-limiting diarrhea (Winter et al., 2010; Chousalkar et al., 2018). It is estimated that gastroenteritis caused by *Salmonella* spp. accounts for 93.8 million cases and 155,000 deaths worldwide each year (Majowicz et al., 2010; Chousalkar et al., 2018). Generally, contaminated food products of animal origin, particularly egg and egg products, are frequently involved in outbreaks of human salmonellosis (Chousalkar and Gole 2016). *Salmonella* Enteritidis and *Salmonella* Typhimurium of various phage types have dominated the epidemiology of *Salmonella* and are the most common causes of human salmonellosis (Hendriksen et al., 2011). *S.* Enteritidis is a predominant serovar isolated from eggshell and egg contents and has been involved in foodborne outbreaks in the USA and UK associated with egg and egg products (Martelli and Davies 2012; Chousalkar et al., 2018).

The genera *Salmonella* is a member of *Enterobacteriaceae* family, and it includes Gram-negative, flagellated, non-sporulating, and facultative bacteria that grow well between 35 and 37°C (Ricke et al., 2013; Heredia and García, 2018). Members of *Salmonella* are commonly classified in 2,579 serotypes according to the Kauffman-White scheme, considering differences in flagellar (H), capsular (K), and somatic (O) antigens (Lamas et al., 2018). Additionally, *Salmonella* serotypes can be subdivided

by molecular subtyping methods or by phage typing (Ricke et al., 2013). This bacterium has the ability to induce localized gastroenteritis in humans and some animals, but the range of infections in the host varies depending on bacterial virulence factors and the immunity and host-resistance capability. The signs and symptoms could evolve from nausea, vomiting, and diarrhea to septicemia or bacteremia, and reactive arthritis as a post-infection sequela has been reported (Ricke et al., 2013; Heredia and García, 2018). *S. enterica* is divided into 6 subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*) because of the differences in biochemical characteristics (Grimont and Weill, 2007). The subspecies *enterica* is responsible for more than 99% of human salmonellosis, and it includes 1,531 serotypes among which are *Salmonella* Typhimurium and *Salmonella* Enteritidis (Lamas et al., 2018; Heredia and García, 2018). *S. enterica*, subsp. enterica serotypes, are principally related to warm-blooded animals whereas the other non-enterica subspecies are more related to cold-blooded animals, although some exceptions have been found (Lamas et al., 2018).

The transmission of non-typhoid *Salmonella* infection to humans can occur through the ingestion of food or water contaminated with waste of infected animals, by direct contact with infected animals or by consumption of food from infected animals (Eng et al., 2015; Heredia and García, 2018). This bacterium has been isolated from a wide range of animals: poultry, sheep, pigs, fish, and seafood and their food products, and also from some other cold-blooded animals (Nguyen et al., 2016; Flockhart et al., 2017, Zajac et al., 2016). Traditionally poultry, meat products, and eggs are the food sources most commonly identified as responsible for outbreaks of salmonellosis (Sanchez et al., 2002; Heredia and García, 2018), although the microorganism has also been found in other foodstuffs. Because *Salmonella* Enteritidis is major causes of salmonellosis in humans from the consumption of infected meat and eggs, understanding the mechanism of enteric infection and innate immune resistance to *Salmonella* has been considered to be worthy of greater more attention.

1.1 Salmonella prevalence and dominant serotypes in China

In China, food poisoning cases are closely related to *Salmonella* infection. Of the 57612 cases reported in China during 1994-2005, *Salmonella* was the most frequently identified agent, accounting for 22.16% of illnesses, followed by Vibrio parahaemolyticus (18.73%), Proteus (11.56%), a mixture of bacteria (11.2%) and Bacillus cereus (9.97%) (Wang et al., 2007). The contamination of food with *Salmonella* causes occasional outbreaks of *S.* Enteritidis. Liu et al. reported a massive outbreak of *S.* Enteritidis linked to *Salmonella*-contaminated eggs, where 197 of about 2000 workers became ill after eating cakes that had been in contact with raw eggs contaminated with *Salmonella* (Liu et al., 2006). Wu et al. reported four serotypes (*S*. Enteritidis, *S*. Typhimurium, *S*. Derby and *S*. Indiana) were commonly found to be transmitted among both food animals and human in China during the period 2003-2011 (Wu et al., 2018).

Up to now, more than 2700 *Salmonella* serotypes have been identified (Mezal et al., 2014). *Salmonella* Enteritidis and Typhimurium were recognized as the most prevalent serotypes associated with infections over the world (Mohamed et al., 2013;

Lai et al., 2014; Maka et al., 2014; Wang et al., 2015). Similarly, based on previous surveillance data, the three dominant serotypes widely distributed in China were *Salmonella* Enteritidis, Typhimurium, and Indiana (Xia et al., 2009; Gong et al., 2014; Zhao et al., 2016).

A sampling survey of *Salmonella* contamination in poultry farms, hatcheries and slaughterhouses in four provinces of China in 2012 found that *S.* Enteritidis (54.7%) and and *S.* Indiana (24.65%) were dominant serotypes (Li et al., 2018). Another survey of integrated broiler chicken supply chain in Qingdao city (China) showed that the *Salmonella* positive rate was 14.9 %, and the predominant serotype was *S.* Enteritidis (69%), followed by *S.* Infantis (10.7%), *S.* Gueuletapee (9.5%), and *S.* Derby (7.1%) (Cui et al., 2016). In addition, investigation of retail foods including chicken, beef, fish, pork, dumplings, and cold dishes in eight provinces in China from 2007 to 2012 (except 2009) also found that a total of 129 *Salmonella* serotypes were detected among 1491 isolates. *S.* Enteritidis (21.5%), Typhimurium (11.0%), Indiana (10.8%), Thompson (5.4%), Derby (5.1%), Agona (3.8%), and Shubra (3.0%) were the seven most important serotypes (Wang et al., 2017). Although the prevalence of different *Salmonella* serotypes in animals and food products showed that *Salmonella* prevalence rate in China varies wildly between different regions (see Table 1-1), *S.* Enteritidis is the one most worthy of attention for public health.

Table 1-1. Investigation of *Salmonella* serotypes and prevalence in poultry and animal food products in China

Years of survey	Samples	Positive	Source	Region	Dominant serotypes	Reference
2017	627	30.10%	Caecal contents; chicken carcasses; chicken meat products in slaughterhouses	Sichuan province	*S.* Enteritidis (69.84%) and *S.* Typhimurium (15.34%)	Zhu et al. (2017)
2016	1148	14.90%	Breeder farms, broiler farms, abattoir, and retail markets	Qingdao city	*S.* Enteritidis (69.0%), *S.* Infantis (10.7%), *S.* Gueuletapee (9.5%), *S.* Derby (7.1%).	Cui et al. (2016)
Mar- July, 2016	1000	14.30%	Fresh fecal swabs from pig and chicken slaughterhouses	Shandong province	Chickens: *S.* Indiana (71.3%) and Enteritidis (24.5%). Pigs: *Salmonella* Rissen (57.1%) and Typhimurium (28.6%).	Zhao et al. (2017)

Aug - Nov, 2015	300	12.70%	Fresh fecal swabs from free-range chicken farms	Shandong province	*S*. Enteritidis (81.6%), *S*. Indiana (13.2%) and *S*. Typhimurium (5.3%).	Zhao et al. (2016)
2012	11592	14.82%	Swabs from Chicken breeding farms and hatcheries; broiler farms; slaughterhouses; distribution and retail stores	Henan, Jiangsu, Heilongjiang and Shandong provinces	*S*. Enteritidis (54.7%) and *S*. Indiana (24.65%).	Li et al. (2018)
2011-2012	495	18.80%	Raw chickens, eggs, and vegetables in the market	Yangzhou city	*Salmonella* Indiana (25.0%), *S*. Typhimurium (21.4%) and *S*. Enteritidis (17.9%)	Li et al. (2017)
2011-2012	2185	11.35%	Cloacal and anal swabs were collected from healthy animals on farms. chickens, pigs, and dairy cows	Henan, Hubei, and Hunan provinces	Chickens: Typhimurium and Enteritidis; Pigs: IIIb and Typhimurium; Dairy cows: Typhimurium and Agona	Hui (2015)
2009 and 2012	1825	19.83%	Faeces, caecal contents and carcasses of chickens	Eight important farming cities in Shandong province	Enteritidis (81.2%) and Indiana (12.4%).	Lai et al. (2014)
	445	11.91%	Faeces, caecal contents and carcasses of ducks		Cremieu (47.2%), Indiana (24.5%) and Typhimurium (9, 17%)	
	692	7.22%	Rectal swabs of pigs		Derby (58%), Typhimurium (18%), and Enteritidis (12%)	
2006-2012	3566	9.10%	Rectal swab samples of chicken, duck, goose, pigeon and Turkey	51 poultry farms in seven regions of 12 provinces of China	*S*. Pullorum (17%), *S*. Typhimurium (15.5%), *S*. Enteritidis (12.1%) and *S* . Indiana (7.7%)	Gong et al. (2014)
2007-2008	764	31.00%	Meat samples (chicken, pork, beef and lamb) from	Shaanxi Province	*S*. Enteritidis (31.5%), *S*. Typhimurium (13.4%), *S*. Shubra (10.0%), *S*. Indiana	Yang et al. (2010)

			supermarkets and free markets		(9.7%), and *S.* Derby (9.5%)	
2008	608	25.0%	Retail food samples: chicken, cold dishes, fresh milk, pork, vegetable, etc.	Five cities in Henan Province	*S.* Enteritidis (34.2%), *S.* Indiana (9.9%), *S.* Derby (9.9%), *S.* Agona (6.6%), and *S.* Typhimurium (5.3%),	Yang et al. (2013)

1.2 Interaction between the avian host and Salmonella infections

The immune system of chickens, like that of other vertebrates, encompasses both innate and adaptive immunity. Innate immunity provides the first barrier against any infection. Adaptive immunity, however, includes responses correlated to lymphocytes that are active during and after exposure to an antigen. The avian host immune system plays a significant interactive role, mainly in three distinct phases during *Salmonella* infection. The first is from the gastrointestinal tract serving in a barrier function. The second phase is gut-associated lymphoid tissue activating the host immune systems. Finally, infection may be cleared by the immune response, or the bird may succumb to the infection, or a subclinical carrier state may develop (Chappell et al., 2009).

The chicken intestine has specialized epithelial cells that initiate innate immunity against infection by producing enzymes and releasing chemokines and cytokines to attract macrophages, granolocytes and immature dendritic cells (Van Immerseel et al., 2002; Wigley, 2014). When innate immune cells cannot completely eliminate *Salmonella*, the adaptive immune responses including cell-mediated and humoral immunity are initiated (Beal et al., 2006). The adaptive immune system eliminates the pathogens in two ways: one through the production of immunoglobulins by B cells, referred to as the humoral immune response, and the other through cellular immune responses performed by T cells (Kean et al., 1994; Cheema et al., 2003).

Salmonella are picked up by macrophages and dendritic cells following the invasion of the gastrointestinal tract (GIT) and then delivered to the spleen and liver (Wigley 2014). The interaction between *Salmonella* and macrophages plays an important role in the progress of systemic diseases in both mammals and birds (Barrow et al., 1994). Macrophages generally destroy the pathogens, though *Salmonella* can adapt to this phagocytic property of macrophages particularly that of the SPI-2 type III secretory system (Hensel, 2000). The SPI-2 system secretes some factors that enter the host cell via phagocytic vacuoles of the macrophages. These effectors interrupt the activity of lysosomes by preventing the fusion of phagosomes, cytokine secretion and the major histocompatibility complex (MHC) (Cheminay et al., 2005). However, the mechanisms of the *Salmonella* intracellular escaping from

the avian immune response and transmission mechanism of bacteria-carrier in chicken remain unresolved.

1.3 Immune-related genes expression in Salmonella infection

Pathogens can influence the expression of genes in the body of hosts by a variety of strategies. Bacteria can modulate the signaling pathway of the host immune system to survive in the host cells (Hossain et al., 2006). *Salmonella* infection in chickens can cause mild enteric inflammation characterized by increased mRNA expression of proinflammatory cytokines including interleukin-6 (*IL-6*), *IL-8*, *IL-12*, LPS-induced tumor necrosis alpha factor (*LITAF*), and interferon gamma (*IFN-γ*) (Kaiser et al., 2000; Withanage et al., 2005; Haghighi et al., 2008; Higgins et al., 2011; Setta et al., 2012; Raehtz et al., 2018). These proinflammatory cytokines were found increased in chicken peripheral immune organ (e.g., caecal tonsils and spleen), intestine and cells (heterophils and macrophages).

During the early stages of Salmonella infection, the innate immunity system including macrophages, granulocytes and immature dendritic cells is activated. A key component of the innate response is the pattern recognition receptors (PRRs), particularly the toll-like receptors (TLR) which recognize highly conserved structural motifs of certain microbes in young chickens (Werling and Coffey, 2007). Up to now，10 TLRs were identified in chicken. The expression of chicken TLRs (e.g., TLR4 and TLR5) has been detected in a broad range of tissues and cell type, but is highly expressed in macrophages and heterophils (Iqbal et al., 2005a; Kogut et al., 2005; Leveque et al., 2003). Significant increases of TLR5 and TLR15 expression were observed in the cecum of chickens after infection with *Salmonella* (Shaughnessy et al., 2009). MacKinnon et al. (2009) showed, however, TLR expression was higher in the distal intestinal segments and tended to increase with *S.* Enteriditis infection, with the exception of TLR 5, 7, and 21. If *Salmonella* are still able to survive, T cells are recruited to the avian gut mucosa (Berndt et al., 2007; Van Hemert, 2007).

Early expression of cytokines, chemokines and apoptotic molecules in the chicken intestine has been reported in previous studies (Cheeseman et al., 2007; Van Hemert 2007; Tohidi et al., 2014). The number of $CD4^+$ T-cells did not increase 1, 5 and 7 days pi with *S.* Enteriditis but the number of $CD8^+$ T-cells increased at 5 and 7 days pi. Macrophages alone exhibited a higher activity in the infected birds on day 1 pi (Van Hemert, 2007). In hatched chicks just infected orally with *Salmonella*, neither $CD4^+$ nor γδ T-lymphocytes were found to be elevated in number in the jejunum; in contrast, jejunal $CD8^+$ cells increased in response to *Salmonella* (Schokker et al., 2010). However, that $CD4^+$ T-cells increased on day 7 pi in the thymus of one-day-old chickens inoculated with *S.* Enteriditis phage type 4 (Asheg et al., 2003) and there was no significant increase in the number of $CD4^+$ and $CD8^+$ T cells from day 7 to 27 pi in the bursa of Fabricius. Inducible immune responsive gene 1(*IRG1*), serum amyloid A (*SAA*), extracellular fatty acid binding protein (*ExFABP*), serine protease inhibitor (*SERPINB10*), trapping 6-like (*TRAP6*), calprotectin (*MRP126*), mitochondrial ES1 protein homolog (*ES1*), interferon-induced protein with tetratricopeptide repeats 5 (*IFIT5*), avidin (*AVD*) and transglutaminase 4 (*TGM4*) are

the other functional genes that are highly induced after *S.* Enteriditis infection in the cecum (Rychlik et al., 2014). Previous studies indicated that the kind and level of gene expression in the spleen and liver may differ from those expressed in the cecum. Interferon γ transcripts were higher in the spleen than in the cecum of chickens at 18 hpi after inoculation with *S.* Enteriditis (Cheeseman et al., 2007). Specific transcripts differ as natural resistance-associated macrophage protein I (*NRAMP1*) was commonly increased in both the cecum and spleen of SE-challenged chickens 48 h pi, whereas *TLR4*, *IL-8* and *IFNγ* were only up-regulated in the cecum at this timepoint (Tohidi et al., 2013b).

Several studies have shown that the level of gene expression in the immune system differs between susceptible and resistant chickens. Expression of *IL-2*, *IL-6*, *IL-8* and *IFNγ* in the small intestine of chickens from a resistant line exceeded that in a susceptible line of birds (Rebel et al., 2005). The level of *IL-6*, *IL-8* and *IL-18* mRNA in heterophils increased after resistant chickens were exposed to *Salmonella*, compared to susceptible chickens. Inversely, the level of anti-inflammatory cytokine transforming growth factor-β4 (TGF-β4), an anti-inflammatory cytokine, decreased in heterophils derived from resistant chickens. Lower expression of *IFNγ* was observed in chickens susceptible to *Salmonella* than in resistant ones (Ferro et al., 2004; Swaggerty et al., 2004). Differences in gene expression depend not only on the presence of pathogens, but also on the status of the animal (e.g. age). Young chickens from a susceptible line, under control conditions, had high levels of *defensin* transcripts (Sadeyen et al., 2004).

Many more genes have been analyzed with the advent of high-throughput techniques. Some cytokines, chemokines and inducible nitric oxide synthase (*iNOS*) were up-regulated 1 dpi with SE and *S.* Typhimurium in the cecum of newly hatched chicks. *Interferon γ*, *IL-8*, *IL-12*, *IL-18*, *IL7Rα*, *MIP-1β*, *iNOS* and *LITAF* were up-regulated in the caecum of the infected chicks 1 day pi and commonly peaked at 2 days pi. Conversely, *Fas* and B-cell lymphoma family (*Bcl-x*) were down-regulated 4 days after infection (Berndt et al., 2007). Interestingly the genes involved in the pro-inflammatory response as well as apoptosis have an important role in initiating immune functions against *Salmonella*. *IL-8*, Chemokine (C-C motif) ligand (*CCL4*), CXC chemokine receptors (*CXCR4*), *TNF receptor*, Caspase 1, 2 and 8, Interferon regulatory factor1 (*IRF1*), *IRF4*, IL-1 receptor-associated kinase, *TGF-β3* and *IL-1β* have been shown to increase in the spleen of SE-infected chickens using microarray analysis (Withanage et al., 2004; Zhou and Lamont, 2007). The up-regulation of *IL-8*, *CCL4*, *LITAF* and *iNOS* within 12 h pi with *S.* Typhimurium was consistent with the role of pro-inflammatory cytokines and macrophages in eliminating *Salmonella* during the early stages of infection (Withanage et al., 2004; Cheeseman et al., 2007; Rychlik et al., 2014). Data from microarray analyses revealed that 309 and 352 genes were significantly transcribed 12 and 24 h pi with SE in chicken ceca (Higgins et al., 2011). Microarray analysis of mRNA transcripts in heterophils isolated from 6-day-old chickens that were challenged with *S.* Enteriditis revealed that 115 and 48 genes were up-regulated and 37 and 125 down-regulated in two different lines (A and B), respectively. Relative expression of some genes such as *IL-6*, *CCL4*, *IL-8* and *K60* changed more than seven-fold in infected heterophils, whereas *TLR7*, Inhibitor of

nuclear factor kappa-B kinase subunit epsilon (*IKBKE*) and immunoglobulin-like receptor B4 were down-regulated more than four-fold (Chiang et al., 2008). The advantage of investigating the gene response *in vivo* as above over infection of cell lines *in vitro* is that the physiological context is retained. This context is important in identifying complex cellular regulation. Luan et al. (2012) divided the 588 genes screened by the Agilent microarray into different categories according to their functions; the genes that were identified to be up-regulated in the intestine in response to *Salmonella* infection were involved in different stages of the immune responses in case and control, such as *TLR5*, Thrombospondin 1 (*THBS1*), *KIT* and Fibroblast growth factor 10 (*FGF10*). Using a combination of methods including 454 pyro-sequencing, protein mass spectrometry and quantitative real-time PCR, Matulova et al. (2013) identified several keyup-regulated chicken genes in spleen after SE infection (see Table 2-2). Splenic gene expression profile after *S.* Enteriditis inoculation in egg-type chickens at the onset of egg-laying was investigated by using Agilent 44 K chicken microarray (Wu et al., 2015). The complicated interaction between the immune system and metabolism at the onset of lay in layer-type chickens influences the immune responses to SE inoculation at 14 dpi. Functional annotation revealed that several GO terms related to immunity were significantly enriched in the inoculated over the non-inoculated control birds at 14 dpi, but not at 7 dpi. Tumor necrosis factor ligand superfamily member 8 (*TNFSF8*), cluster of differentiation (*CD*) *86*, *CD274*, *BLB1* (MHC class II β chain) and *BLB2* play important roles in the response to *S.* Enteriditis inoculation (Wu et al., 2015). The immune-related genes during *S.* Enteriditis infection are listed in Table 1-2.

Table 1-2. List of key genes associated with *Salmonella* Enteritidis infection in chickens.

Technology means	Organ	Breed	Day of age	DPI	regulation of genes	Ref.
Microarray	Spleen	Intercross lines	1	7 or 8	Gag protein, *IL-8*, *CCL4*, *CXCR4*, TNF recepror, *Caspase 1*, *2* and *8*, *IRF1*, *IRF4*, IL-1 receptor-associated kinase, *TGFβ3*, *IL-1β*	Zhou and Lamont (2007)
Real-time PCR	Caecum	Native chickens	1	2	*TLR4*, *IL-8*, *INF*g, *NRAMP1*	Tohidi et al. (2013b)
44k microarry	spleen	Egg-type chicken	140	14	*Glucocorticoid*, *TNFSF8*, *CD86*, *CD274*, *BLB1*, *BLB2*	Wu et al. (2015)
Real-time PCR	Caecum	SPF White Leghorn	1	1 or 2	*INFγ*, *IL-8*, *IL-12*, *IL-18*, *IL-7Rα*, *CCL4*, *iNOS*, *LITAF*	Berndt et al. (2007)
Real-time PCR	Caecum	SPF White Leghorn	1	4	*IL-2*, *Fas*↓, *Bcl-x*↓	Berndt et al. (2007)
44k microarry	Heterophils	Peripheral blood of broiler chickens	6	1 hour	*IL-6*, *CCL4*, *IL-8*, *K60*, *TLR7*↓, *IKBKE*↓	Chiang et al. (2008)
Chips	jejunum	Native chickens	14	1	*TLR5*, *FGF10*, *KIT*, *THBS1*, *IL-8*↓, *CD79B*↓	Luan et al. (2012)
Pyrosequencing	spleen	Brown chickens	42	4	*AVD*, *ExFABP*, *IRG1*, *AH211*, *TRAF6*, *SAA*	Matulova et al. (2013)
Combination of pyrosequencing, protein mass spectrometry and real-time PCR	Caecum	Brown chickens	1	4	IgG, *IRG1*, *SAA*, *ExFABP*, *IL-22*, *TRAP6*, *MRP126*, *IFNγ*, *iNOS*, *ES1*, *IL-1β*, LYG2, IFIT5, *IL-17*, *AVD*, *AH221*, SERPINB	Matulova et al. (2013)
Real-time PCR	spleen	Broiler, Leghorn and Fayoumi	1	2 or 18 hours	*IL-18*, *INFg*	Cheeseman et al. (2007)

Note: ↓ means down-regulation; no ↓ means up-regulation.

2 The role of microRNA in *Salmonella* infection

MicroRNAs (miRNAs) are a class of genome-encoded small RNAs that regulate eukaryotic gene expression at the post-transcriptional level, by repressing target transcripts containing partially or fully complementary binding sites, mainly present in the 3'-untranslated region (UTR) and coding sequence of mRNAs (Bartel, 2004; Ghildiyal and Zamore, 2009; Maudet et al., 2014a). miRNAs are highly evolutionarily conserved sequences, expressed in a variety of tissues and cells, and play important roles in various physiological and pathologic processes. Increasing evidence suggest roles for miRNAs in bacterial infectious diseases by modulating inflammatory responses, cell penetration, tissue remodeling, and innate and adaptive immunity (Zhou et al., 2018).

Many studies have shown that the abnormal regulation of miRNAs contribute to disease pathogenesis. Modulation of the host miRNA repertoire by *Salmonella* infection was first described in mouse macrophages, where the NF-κB-dependent miRNAs miR-155, miR-146a/b and miR-21, were shown to be strongly induced upon infection (Maudet et al., 2014a; Schulte et al., 2011). Members of the let-7 miRNA family were down-regulated upon *Salmonella* challenge both in macrophages and in epithelial cells (Schulte et al., 2011), suggesting that repression of this miRNA family constitutes a common signature of the infection of phagocytic and non-phagocytic cells by *Salmonella* (Maudet et al., 2014b). The let-7 family repress the transcript abundance of the key major immunomodulatory cytokines IL-6 and IL-10 (Liu et al., 2011; Schulte et al., 2011), and therefore let-7 down-regulation upon *Salmonella* infection increases IL-6 and IL-10, likely contributing to a balanced host inflammatory response. Hoeke et al. (2013) showed a rapid increase in miR-29a in the ileum at 3h post-infection of *Salmonella*-infected piglets. Further research showed that intestinal *Salmonella* typhimurium infection leads to miR-29a-induced decreased regulation of *Caveolin 2*, a focal adhesion factor that is involved in recognition of bacterial pathogens and modulates the activation state of the small Rho GTPase CDC42 (Hoeke et al., 2013). By a combination of high-throughput screening with a library of miRNA mimics and RNA-seq, Maudet et al. (2014b) suggested that miRNAs are potential modulators in *S.* Typhimurium infection and that distinct miRNAs impair various infection stages. They further found that down-regulation of the miR-15 family up-regulated *cyclin D1* expression upon *Salmonella* infection and G2/M arrest of host cells dramatically increased *Salmonella* replication within hosts (Maudet et al., 2014b; Zhou et al., 2018).

Virulent *S.* Enteritidis promotes miR-128 levels in mouse intestinal epithelial, which, in turn, decreases the epithelia-secreted M-CSF and inhibits the subsequent recruitment of macrophage (Zhang et al., 2014). *Salmonella*-challenged pigs showed down-regulated miR-214 expression and up-regulated miR-331-3p expression in whole blood. While levels of the candidate targets of miR-214 (*SLC11A1* and *PIGE-108A11.3*) were increased following challenge, the potential target (*VAV2*) of miR-331-3p was reduced (Bao et al., 2015; Zhou et al., 2018). Another report found that miR-143 and miR-26 responsible for dysregulated mRNAs might be involved in the progression of *Salmonella* infection by binding site enrichment analysis in pig whole

blood (Yao et al., 2016). Latest research has shown that miR-143 could inhibit intracellular *S.* Typhimurium proliferation by its target *ATP6V1A* in porcine macrophages (Huang et al., 2018). The results provide new insights that miR-143 may play key roles in intracellular interactions between *Salmonella* and the host immune system. In zebrafish embryos, miR-146a/b down-regulation by *S.* Typhimurium infection up-regulated the apolipoprotein genes that have been previously linked to immunoregulation and host defense (Khovidhunkit et al., 2004; Ordas et al., 2013). Moreover, vaccination of miR-155-deficient mice with an attenuated vaccine against *S.* Typhimurium failed to protect them against virulent *S.* Typhimurium (Rodriguez et al., 2007; Zhou et al., 2018). It is suggested miR-155 regulates the function of lymphocytes and dendritic cells (DC), leading to an overall decrease in immune responses. As part of the present research it was observed, we observed that up-regulation of the gga-miR-155 down-regulated *LRRC59* expression upon *S.* Enteritidis infection in young chicken (Li et al., 2017). It is also suggested that miR-155 plays an important regulatory role in the innate immune response to *Salmonella* infection in animals. Thus, these dysregulated miRNAs may be functionally important for manipulating *Salmonella*-induced inflammation.

During *Salmonella* infection, cells recognize invading pathogens through several conserved pattern-recognition receptors (PRRs), which include the Toll-like receptors (TLRs) and Nod-like receptors (NLRs) that are located on the cell membrane or in the cytoplasm, respectively (Eulalio et al., 2012; Kawai and Akira, 2011). After recognizing the pathogen-associated molecular patterns (PAMPs) of *Salmonella* such as lipopolysaccharide (LPS), TLRs can transduce downstream signaling through either MyD88 or TRIF. Regulation of the TLR signaling pathway by miRNAs is shown in Figure 1. A role for miRNAs in the response of eukaryotic cells to bacterial infections was originally inferred from experiments involving the sensing of purified PAMPs by TLRs. miRNAs have emerged as important controllers of TLR signaling (O'Neill et al., 2011), for example, LPS stimulation of TLR4 and downstream NF-κB activity induced by miR-146, miR-147, and miR-155 (Ceppi et al., 2009; Liu et al., 2009; O'Connell et al., 2007; Taganov et al., 2006; Tili et al., 2007).

Upon infection, miR-155 and miR-146 are two miRNAs induced by the NF-κB pathway through PRR sensing of pathogen motifs, in particular LPS (Duval et al., 2017). miR-146 acts as an anti-inflammatory regulator, by directly targeting *TRAF6* (TNF Receptor-associated factor 6) and *IRAK1* (IL-1R-associated kinase 1) resulting in tolerance to low doses of LPS (Staedel and Darfeuille, 2013; Taganov et al., 2006).

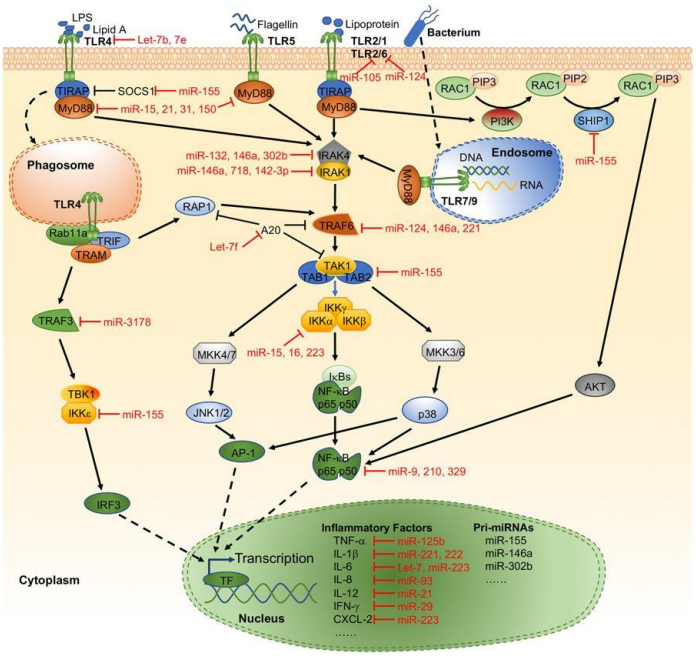

Figure 1-1: Representative miRNAs in the regulation of TLR signaling. TLRs recognize different bacterial components and induce NF-κB signaling or activate other transcription factors through adapter molecules and downstream signaling molecules. Different transcription factors then initiate transcription of various inflammatory factors. The transcription of miRNAs is most commonly mediated by RNA polymerase II, under the control of transcription factors, and transcripts are then processed by two nucleases, Drosha and Dicer. The mature miRNAs will then be incorporated into the RNA-induced silencing complex (RISC) and guide the RISC to their target mRNA(s) in cytoplasm. Both early- and late-phase-activated TLRs induce different types and expression levels of inflammatory factors and miRNAs. This figure is reproduced from Zhou et al. (2018).

In contrast, miR-155 is induced by higher doses of LPS, at levels which result in pro-inflammatory NF-κB activity, as well as by TNF-α and interferon β, via TAB2 (Duval et al., 2017; O'Connell et al., 2007). miR-155 can fine-tune the TLR signaling pathway through direct targets MyD88, an adaptor protein that play pivotal role in linking up- and down-stream protein molecules in signaling. In addition, miR-155 also targets *SHIP1*, a negative regulator of the NF-κB pathway (Cremer et al., 2009; O'Connell et al., 2009; Testa et al., 2017), and *SOCS1* (suppressor of cytokine signaling 1), an effector involved in the homeostasis of regulatory T cells (Lu et al., 2009; Wang et al., 2018). These all show that miRNAs have excellent regulatory function in host immune response to pathogens, such as *Salmonella.*

3. The role of FOS in *Salmonella* infection

The term prebiotics was introduced at the end of the twentieth century and originally was defined as a "selectively fermented ingredient that induces specific changes in the composition and/or the intestinal flora, conferring benefits to health and well-being of the host" (Gibson and Roberfroid, 1995; Kareb and Aider, 2018). In recent years, there is a great deal of interest in the use of prebiotics as functional food ingredients or feed additives as they confer health benefits in humans and animals (Al-Sheraji et al., 2013; Kareb and Aider, 2018). Prebiotics are found in several vegetables and fruits (e.g., chicory and banana) and are considered to be 'functional food' components which contain biologically active compounds that have documented clinical benefits on health, ranging from the prevention of colorectal cancer to the regulation of host defense against viral and bacterial infections by changing the interaction between pathogenic and beneficial bacteria (Gibson and Roberfroid, 1995; Hardy et al., 2013; Roberfroid et al., 2010). Prebiotics pass undigested through the small intestine to reach the distal intestine where they are available for probiotic bacteria without being used by other gut bacteria. The beneficial effects of prebiotics include an increase of desirable bacteria and a decrease of detrimental bacteria in the gut, alleviation and treatment of gastro-enteritis, prevention of infections, increased absorption of minerals, regulation of blood lipids, and reduction of cancer risk (Al-Sheraji et al., 2013). Various potential mechanisms have been proposed for the health benefits of prebiotics (Figure 2.2). These include: i) providing substrates for intestinal commensal microbiota affecting their growth and metabolic activities (Saulnier et al., 2009); ii) preventing adhesion of certain bacterial species by occupying carbohydrate binding sites in bacteria and host cells (Roberfroid et al., 2010); iii) increase in intestinal production of short-chain fatty acids (SCFA), and enhancing their binding to G-coupled protein receptors or other immune receptors and affecting immunomodulation and host metabolism (Covington et al., 2006; Delzenne et al. 2005; Kelly 2008); and iv) exerts interactions with carbohydrate receptors on immune cells and ligates specific immune receptors (e.g., C-type lectin receptors (CLRs) and TLRs) (Chermesh et al., 2007; Vogt et al., 2015).

In general terms, the immunomodulation by prebiotics is achieved through indirect and direct mechanisms. Indirect mechanisms mainly involve favoring expansion of lactic acid bacteria (e.g., *bifidobacteria*) and production of SCFA (including acetate, propionate, and, especially, butyrate). A large number of experimental studies, especially in healthy animals and humans, indicate prebiotics (e.g., inulin or fructooligosaccharides) supplementation will generally result in increased *Bifidobacteria* numbers (Bouhnik et al., 2006; Buddington et al., 1996; Neyrinck et al., 2011; Tzortzis et al., 2005), increased levels of fecal or mucosa sIgA (Hosono et al., 2003; Min et al., 2016; Roller et al., 2004; Swanson et al., 2002), increased levels of pro-inflammatory factors (Hosono et al., 2003, Pié et al., 2007; Zenhom et al., 2011), as well as increased activity of different immune cells (Akhter et al., 2015; Benyacoub et al., 2008; Dwivedi et al., 2016). Several studies, however, found inconsistencies in the efficacy of prebiotics (Zhang et al., 2003; Jiang et al., 2006; Biggs et al., 2007). This discrepancy may reflect differences in laboratory methods,

experimental conditions, and variation in the gut microbiota of individual animals (Kelly. 2008; Kim et al. 2007, Bouhnik et al., 2006; Vogt et al., 2015). SCFA are produced by microbial fermentation in the distal intestinal segments, colon and cecum. In addition, these SCFA are rapidly transferred to the

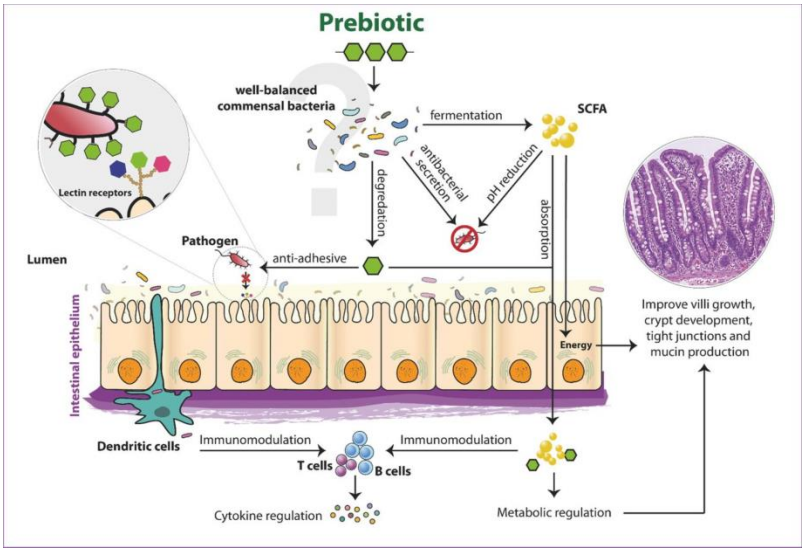

Figure 1-2: Potential mechanisms of action of prebiotics. Prebiotics are metabolized by the gut commensal microbiota. The gut microbiota can ferment prebiotics into short-chain fatty acids (SFCA), mainly acetate, propionate, and butyrate. SCFA lower the luminal pH, provide energy sources for epithelial cells, and have profound effects on inflammation modulators and metabolic regulations. A well-balanced bacterial community can also improve intestinal mucosal structure. Some bacterial strains produce antimicrobial factors or stimulate the immune system by signaling dendritic cells. Oligosaccharides and monosaccharides can reduce pathogen colonization by blocking the receptor sites used by pathogens for attachment to the epithelial cell surface. This figure is reproduced from Pourabedin & Zhao (2015).

bloodstream (Sembries et al., 2003) and can activate and induce G protein-coupled receptor-signal transduction (GPR) on immune cells (Covington et al., 2006). For instance, the GPR41 receptor is preferentially liganded by butyrate and isobutyrate (Le Poul et al., 2003); while for the GPR43, acetate and propionate are the most potent ligands (Brown et al., 2003; Nilsson et al., 2003). SCFA may affect leucocytes within the gut-associated lymphoid tissue (GALT). Femia et al. (2002) found that cecal SCFA especially butyrate were significantly increased by long-term supplementation of rats with FOS-inulin. Butyrate, however, is known to suppress lymphocyte proliferation, to inhibit cytokine production of Th1-lymphocytes, to inhibit NF-κB activation, to induce T-lymphocyte apoptosis and NK cell cytotoxicity (Säemann et al., 2000; Cavaglieri et al., 2003; Pratt et al., 1996; Watzl et al., 2005). In addition, the presence of SCFA in the intestines contributes to a lower pH, a better

bioavailability of calcium and magnesium, and inhibition of potentially harmful bacteria (Teitelbaum and Walker, 2002, Wong et al., 2006). Mechanisms of direct immunomodulation by prebiotics are thought to entail liganding of PRRs on the surface of epithelial cells and immune cells (Vogt et al., 2015). PRRs include the well-known TLRs, the membrane-bound CLRs, the cytosolic proteins such as NOD like receptors (NLRs) and RIG-I-like receptors (RLRs) (Osorio and e Sousa, 2011, Loo and Gale Jr. 2011, Elinav et al., 2011). Possible direct effects of inulin-FOS are thought result from recognition of PRRs on the surface of gut immune cells (DCs and macrophages) and epithelial cells (MacPherson et al., 2004; Abreu. 2010). Recent studies in rat intestinal epithelial cells (Ortega-González et al., 2014) and in rat monocytes (Capitán-Cañadas et al. 2014) showed that non-digestible oligosaccharides (FOS or inulin) are TLR4 ligands and directly modulate production of pro-inflammatory cytokines. In addition, oligosaccharides may possess the capacity of interacting with cell membrane lipids or even inserting in the membrane (Figdor and van Spriel, 2010). Although these hint at prebiotics having the ability to directly influence the intestinal cells, the subsequent consequences and overall function require more examination.

Compared to prebiotic use in humans, that in poultry has just a brief history. Oligosaccharides including fructooligosaccharides (FOS) (Kim et al., 2011, Swiatkiewicz et al., 2011) and mannanoligosaccharides (MOS) (Baurhoo et al., 2009, Xiao et al., 2012) are the most commonly studied prebiotics in chicken production. Similar to studies in humans and rats, oligosaccharide supplementation also inhibits potential pathogens (i.e., *clostridia* and *E. coli*), and increases the abundance of beneficial bacteria (i.e., *Bifidobacterium* and *Lactobacillus*) (Kim et al., 2011, Peinado et al., 2013, Shanmugasundaram et al., 2013). In contrast, other studies have shown little or no significant effect (Rehman et al., 2008; Zhang et al., 2003).

FOS is not hydrolyzed by mammalian or avian digestive enzymes and thereby reaches the colon undigested, where it serves as a fermentation substrate for gut microbiota (Roberfroid et al., 2010). Rehman et al. (2008) showed that inulin addition (10 g/kg diet) did not change microbial community structure in the jejunal and cecal digesta in broilers, whereas Geier et al. (2009) found the ileal microbiota to have obvious differences in FOS-fed broilers (5 g/kg diet), compared to the controls. Kim et al. (2011) indicated that FOS (2.5 g/kg diet) can increase the number of *Lactobacillus* while limiting the proliferation of *C. perfringens* and *E. coli* in broilers. Supplementation of diets with FOS has positive modulatory effects on the immune system in chickens, such as increasing the titers of plasma IgM and IgY (Janardhana et al., 2009), enhancing general resistance (sheep red blood cells and heterophil / lymphocyte ratio) (Emami et al., 2012; Kim et al., 2011), and regulating the level of pro-inflammatory cytokines (Schley and Field, 2002; Shang et al., 2015). Interestingly, in an *in vitro* study, FOS/inulin-treated cells (chicken macrophage-like HD11 cell line) had increased phagocytic ability to kill *S.* Enteritidis and this effect was linked to reduced IL-1β-associated macrophage cell death (Babu et al., 2012). In addition, a gene cluster, called the FOS locus, has been identified in the genome of avian extraintestinal *E. coli* (ExPEC) that encodes proteins involved in FOS metabolism (Schouler et al., 2009; Porcheron et al., 2011). The products of the gene

cluster account for strain differences of ExPEC in colonization of the chicken intestine (Porcheron et al., 2012). It is important for future studies, therefore, to apply high-throughput sequencing techniques and provide a community-wide analysis of the gut microbiota at different levels of the phylogenetic classification when assessing prebiotic supplementation.

MOS has been found to promote growth of beneficial microflora in the gut and to maintain gut health, improve intestinal morphology and enhance host immunity in birds. Paul et al. (2001) first reported that MOS can improve the efficiency of feed conversion in chickens. Diet is the important factor influencing the gut microflora population. Afrouziyeh et al. (2014) showed that proportions of *lactobacilli* and *bifidobacteria* were highest in birds fed 2 and 3g/kg MOS in comparison to birds supplemented with avilamycin. The relative length of the small intestine was affected by the addition of MOS and the effects were dependent on the age of birds as well as the dosage level of MOS (Yang et al., 2007, 2008; Raza et al., 2017). Pourabedin et al. (2013) observed that the length of villa and goblet cell numbers was increased in the ileum and jejunum of MOS-supplemented birds, while Markovic et al. (2009) found the number of goblet cells did not significantly differ among experimental groups. MOS has been assumed to have a direct effect on the immune cells through its mannan moiety. As a mannose receptor, TLR4 recognizes mannan and mannan-associated molecules (Sheng et al., 2006) and then regulates cytokine expression (Singboottra et al. 2006). MOS supplementation was also reported to lower the ileal gene expression of pro-inflammatory cytokines while increasing anti-inflammatory cytokines after challenging broilers with *Escherichia coli* (Wang et al., 2016). Dietary supplementation with YCW (yeast cell wall) / MOS have been reported to modulate immune cell variables (Shanmugasundaram and Selvaraj, 2012) and moderate the inflammatory response (Shanmugasundaram et al., 2013). In addition, higher antibody titers (against influenza, reovirus and NDV) were observed in the blood of MOS-supplemented birds (Gabriela et al., 2009; Hajati and Teimouri, 2014). From all of this, it is suggested that FOS and MOS play a potentially important role in promoting growth performance and antibiotic substitution in poultry.

The control of *Salmonella* in poultry production has been a high priority for the poultry industry. In order to reduce the incidence of *Salmonella* colonization at the farm level, several preventive strategies have been proposed in addition to well-established, regular health and biosafety measures (Vandeplas et al., 2010). Vaccination and feed additives such as organic acids, antibiotics, prebiotics and probiotics are the most widely used control methods that have been investigated in poultry production (Vandeplas et al., 2010). Many studies have suggested prebiotics as being efficient and cost-effective feed additives to inhibit intestinal colonization by enteric pathogens such as *Salmonella*. MOS are the most widely studied oligosaccharides with regard to their activities against *Salmonella*. Spring et al. (2000) investigated the effects of MOS in chickens that were orally challenged with *S.* Typhimurium or *S.* Dublin at 3 days of age. At 7 days after challenge, a significant decrease in cecal bacteria burden was observed when MOS was part of diet (Spring et al., 2000). Similar results were reported when chickens were challenged with *S.*

Enteritidis (Fernandez et al., 2002). In a study on broilers challenged with LPS derived from *Salmonella*, MOS (2 g/kg diet) resulted in a mild immune response that terminated the systemic inflammation earlier than achieved with sub-therapeutic levels of virginiamycin (Baurhoo et al. 2012). Feed supplementation with a specific prebiotic mixture consisting of galactoglucomannan oligosaccharides and arabinoxylan did not limit intestinal colonization of *Salmonella* in broilers challenged with *S.* Typhimurium on day 10 post-hatch (Faber et al., 2012). More recently, MOS increased $CD4^+$ and $CD8^+$ lymphocyte counts in the ileum and cecum, and reduced fecal shedding of *Salmonella* in chickens challenged with *S.* Enteritidis (Lourenço et al., 2015). Other prebiotics have also been studied. When FOS was fed to chickens at the 0.375% or 0.75% levels, little effects on *Salmonella* incidence was observed, however, when birds were stressed by feed and water withdrawal, *Salmonella* level was reduced about four-fold in the cecum of 0.75% FOS treated birds (Bailey et al., 1991). Reduced *Salmonella* colonization was also observed with low-dose (0.1%) feeding of FOS (Fukata et al., 1999). In an *in vitro* study, Babu et al. (2012) investigated the influence of FOS-inulin on the ability of the chicken macrophage-like HD11 cell line to phagocytose and kill *Salmonella* Enteritidis. They found that prebiotic treated cells had significantly fewer viable intracellular *S.* Enteritidis than did the untreated cells, and this effect was linked to reduced IL-1β-associated macrophage cell death. Eeckhaut et al. (2008) evaluated administration of two different doses (0.2% and 0.4%) and chain length (average DP of 3 and 9) of xylo-oligosaccharides (XOS) for 5 weeks on *Salmonella* colonization in chickens experimentally infected with *S.* Enteritidis at 14 days post-hatch. Despite the extent of these studies, little information is available describing the roles of dietary FOS on inflammation, especially in the peripheral immune organs of chicks. Similarly, the effect of a FOS-supplemented diet in potentially alleviating induced inflammation in *Salmonella*-exposed chicks is warranted.

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2

Objectives and dissertation structure

The main objectives of this thesis consisted of two parts:

(1) To identify differentially expressed splenic microRNAs and mRNAs following *Salmonella* Enteritidis infection in chickens using RNA sequencing;

(2) To investigate whether FOS addition alters the expression of inflammatory genes involved in TLR4-MyD88 signaling following *Salmonella* infection.

To achieve these objectives, the research strategy involved developing a more powerful disease model than previously used, transcriptome screening, identification of resistance genes and pathways and, finally, evaluation of FOS, provided by diet to chicks or used on immune cells *in vitro*.

Differentially expressed microRNAs and gene transcripts (mRNAs) in spleen, as well as signaling pathways were investigated in three cohorts of chicks. These consisted of those that were resistant to experimental infection (**R**, SE-challenged with only slight clinical symptoms and blood loads, $< 10^5$ cfu / 10 μL), susceptible to infection (**S**, SE-challenged with severe clinical symptoms and burdens of $> 10^7$ cfu / 10 μL in blood) and control birds (**C**, non-challenged, no SE in blood).

The effects of FOS on regulating the immune response to *Salmonella* Enteritidis infection were also investigated, using both *in vivo* and *in vitro* models. These findings will help facilitate understanding resistance and susceptibility to *Salmonella* infection in the earliest phases of the host immune response in chickens, provide new approaches to develop strategies for *Salmonella* prevention and treatment, and may eventually aid in improving innate resistance by genetic selection.

Outline of thesis

This study is a compilation of scientific papers that have been published or are under review. It is structured as follows:

Chapter 1 is a general introduction and a review of relevant literature on the dissertation topic.

Chapter 2 is Objectives and dissertation structure

Chapter 3 investigates the splenic transcriptome by mRNA sequencing and pathway analysis providing novel insights into the susceptibility to *Salmonella* Enteritidis infection in chickens.
Reference: Messenger RNA sequencing and pathway analysis provide novel insights into the susceptibility to Salmonella Enteritidis infection in chickens. Peng Li et al. (2018), in Front. Genet.

Chapter 4 investigates the splenic microRNA expression profiles and integration analyses involved in the host responses to *Salmonella* Enteritidis infection in chickens.
Reference: Splenic microRNA expression profiles and integration analyses involved in host responses to Salmonella Enteritidis infection in chickens. Peng Li et al. (2017), in Front. Cell. Infect. Microbiol.

Chapter 5 screens the optimum concentration of dietary FOS for reducing the expression of pro-inflammatory factors during *Salmonella* infection in chicken.
Peng Li et al. (2018, submitted in Poultry Science).

Chapter 6 investigates the effects of FOS in modulating the expression of inflammatory genes involved in MyD88-dependent signaling following *Salmonella* infection in young chickens. *Peng Li et al. (2018, preparing to submit)*

Chapter 7 discusses the main findings of the overall research, conclusions, potential improvements and future prospect.

3

Identification of mRNAs and pathways associated with the host response to *Salmonella* infection

The aims of this chapter were to identify the immune-related genes and pathways in Salmonella enterica challenged-susceptible, challenged-resistant and non-challenged chicks using messenger RNA sequencing.

Identical Peng Li, Wenlei Fan, Nadia Everaert, Ranran Liu, Qinghe Li, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. 2018. Messenger RNA sequencing and pathway analysis provide novel insights into the susceptibility to *Salmonella* Enteritidis infection in chickens. *Front. Genet* 9:256.

Abstact : *Salmonella* Enteritidis (SE) is a foodborne pathogen that negatively affects both animal and human health. Controlling poultry SE infection for resistance will have great practical significance for human public health, as poultry are considered to be important sources and carriers of the disease. In this study, the splenic transcriptomes of challenged-susceptible (**S**), challenged-resistant (**R**) and non-challenged (**C**) chicks (3-d old, specific-pathogen-free White Leghorn) were characterized in order to identify the immune-related gene markers and pathways. A total of 934 significant differentially expressed genes (DEGs) were identified in comparisons among the C, R and S birds. First reported here, the DEGs involved in the Forkhead box O (FoxO) signaling pathway, especially FoxO3, were identified as potential markers for host resistance to SE infection. The challenged-susceptible birds exhibited strong activation of the FoxO signaling pathway, which may be a major defect causing immune cell apoptosis as part of SE-induced pathology; these S birds also showed weak activation of mitogen-activated protein kinase (MAPK)-related genes, contrasting with strong splenic activation in the R birds. Interestingly, suppression of several pathways in the immune response against *Salmonella*, including cytokine-cytokine receptor interaction and Jak-STAT, was only found in S birds and there was evidence of cross-talk among these pathways, perhaps contributing to susceptibility to *Salmonella* infection. These findings will help facilitate understanding resistance and susceptibility to SE infection in the earliest phases of the host immune response through *Salmonella*-induced pathways, provide new approaches to develop strategies for SE prevention and treatment, and may enhance innate resistance by genetic selection in animals.

Keywords: Spleen transcriptome; *Salmonella* Enteritidis; Resistant and susceptible; Immune-related genes and pathway; chicken

1. Introduction

Salmonella Enteritidis (SE) is an enteric bacterium that can colonize chickens, contaminating meat and eggs; it does not cause production losses, but birds carry a bacterial burden with non-obvious symptoms, thereby constituting an insidious risk for public health (Barrow et al., 2012; Calenge and Beaumont, 2012; Kogut and Arsenault, 2017). SE is among the top ranking food-borne pathogens causing huge economic and human life losses. Poultry are considered to be important sources and carriers of the disease. Although use of appropriate control measures can reduce Salmonella contamination in poultry, *Salmonella* cases continue (Inns et al., 2015). Control of SE, therefore, is highly desirable from the perspective of both animal and human health. In recent years, genetic selection of birds is considered to be an efficient and permanent way to control *Salmonella* infection (Berthelot et al., 1998; Gou et al., 2012; Kaiser and Lamont, 2001; Kogut et al., 2012; Li et al., 2017b). A better understanding of host immunological response mechanisms should be given priority in achieving this goal.

The main route of SE infection is the oral intake of contaminated feed or water. From the intestinal tract, SE can quickly enter the bloodstream and colonize the internal organs including liver, spleen and heart (Chappell et al., 2009). The spleen plays a major role in detecting cell damage during bacterial infection and in the pathogenic mechanisms of bacterial clearance (Altamura et al., 2001). Increasing evidence suggests that the spleen plays a greater role in immune function in avian than in mammalian species, and is responsible for an immediate immune reaction after recognizing pathogens by filtering antigens from the blood (Smith and Hunt, 2004; Li et al., 2017a).

Although there have been several previous studies focusing on the splenic transcriptome following infection with *Salmonella enterica* (Matulova et al., 2012; Zhou and Lamont, 2007), avian pathogenic *Escherichia coli* (APEC) (Nie et al., 2012; Sandford et al., 2011) and virus (Haq et al., 2010; Smith et al., 2011; Wang et al., 2006), little is known about immune-related genes and pathways between resistant and susceptible birds during the course of SE pathogenesis. This paper identifies genes and pathways that are differently expressed in susceptible versus resistant chickens, after challenged with SE, to aid understanding of host immune resistance to SE infection; an earlier report (Li et al., 2017a) presented differences that were apparent at the miRNA level.

2. Materials and methods

2.1 Ethics Statement

All animal care and experimental procedures were approved by the Institute of

Animal Sciences, Chinese Academy of Agricultural Sciences (approval number: IASCAAS-AE20140615).

2.2 Animals and Sample Collection

Specific-pathogen-free chicks (White Leghorn) were obtained from the Beijing Laboratory Animal Research Center and were treated as described earlier (Gou et al., 2012; Li et al., 2017a). Groups of 30 SE-challenged chicks were initially screened at 0.5, 1, 2, 4, 6 and 8-days post infection at 3 d of age; 24-h post infection was found to be optimal for showing differences (clinical symptoms and bacterial burden) between the 3 groups to best expose potential differences in mRNA expression. The challenged-susceptible (**S**) chicks exhibited severe clinical symptoms (diarrhea, drooping wings and dying) and higher bacterial loads (> 10^7 cfu / 10 μL blood) compared with the others. Chicks with only slight clinical symptoms and lower bacterial loads (< 10^5 cfu / 10 μL blood) were identified as challenged-resistant (**R**) birds. Six challenged chickens conforming to the requirements (3 R and 3 S) were selected from the 30 chickens sampled at 24 h. No *Salmonella* were detected in the PBS-challenged chicks and 3 were randomly chosen from 15 chicks as controls (**C**) at same time-point.

2.3 RNA Extraction, cDNA Library Preparation, and RNA sequencing

Total splenic RNA was extracted from each of the 9 birds, using RNeasy Plus Micro Kit (74034) (Qiagen, Hilden, Germany) following the manufacturer's protocol. The total RNA quantity was evaluated using Bioanalyzer 2100 and RNA Integrity Number (RIN) scores exceeding 8.0. For each sample, approximately 3 μg of total RNA was depleted of ribosomal RNA (Epicentre Ribo-Zero Gold Kit, Illumina, San Diego, CA). Following purification, the RNA fractions were broken into small pieces using divalent cations at high temperature. And the final cDNA library was generated using reverse transcription amplification of cleaved RNA fragments. Sample Preparation Kit (Illumina, San Diego, CA), and paired-end sequencing was performed on an Illumina Hiseq2000 by LC-BIO (LC Sciences, Houston, TX) and 100 bp paired-end reads were generated. Quality control of reads was determined by FastQC software (v0.10.1), details of which were described earlier (Li et al., 2017a). In brief, clear data were obtained from the raw reads, eliminating contamination with sequencing adapters or poly-N and low quality reads (Q values < 20), along with potential residual ribosome RNA. Clean reads were aligned to the reference genome (Gallus gallus 4.0) database using TopHat (Trapnell et al., 2009) software (v2.0.9) and Bowtie (Langmead and Salzberg, 2012) (v2.0.0), and the mapped transcripts were assembled de novo using Cufflinks (Trapnell et al., 2010). The RNA-seq data can be obtained from the BIG Data Center (Nucleic Acids Res 2017) database with the accession number CRA000463.

2.4 Differentially Expressed Genes (DEGs) and Function Enrichment Analysis

Fragments per kilobase of exon per million mapped reads (FPKM) were used to quantify the abundance of mRNAs using the Cufflinks package (v2.1.1). Analysis of DEGs between the 3 groups of chickens was performed with a false discovery rate (FDR < 0.1), $P < 0.05$, and |fold change| > 1.5. The DEGs were used to implement Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis using KOBAS (Xie et al., 2011) (v3.0) and DAVID (Dennis et al., 2003) (v6.8). Volcano plots were performed using OmicShare tools (www.omicshare.com/tools). The normalized read counts of some mRNAs were set to be 0.01 for further calculation if they had no reads in the library.

2.5 Validation of DE Genes by quantitative real-time PCR (qPCR)

To validate the DEGs identified by RNA-seq, qPCR analyses were performed to measure transcript abundance of 16 selected genes (*IL10RB*, *TNFSF10*, *LAMP1*, *ZNF207*, *CCND1*, *GJA1*, *FTH*, *HBBA*, *GAL1*, *CREBBP*, *BRI3BP*, *SOCS1*, *ICOS*, *CTLA4*, *AVD*, and *IL8*) in an ABI 7500 Detection System (Applied Biosystems, Foster, CA). The candidate genes were selected for their involvement in multiple immune response pathways and their levels of differential expression (high, mean FPKM > 1000; middle, 70 < mean FPKM < 200; low, mean FPKM < 50) in the RNA-seq analysis. cDNA was obtained from the same individual samples used in RNA-seq. The qPCR amplification was as follows: each qPCR reaction (20 µl), run in triplicate, consisted of either 1 µl of template cDNA, 10 µl of 2 × KAPA SYBR FAST qPCR Master Mix (Roche, Shanghai, PRC), 0.4 µl ROX Low, 0.5 µl of each primer, and 7.6 µl PCR-grade water. The qPCR program was performed following the instructions of ABI 7500 with default parameters. $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the relative mRNA abundance. *β-actin* was used as the housekeeping gene and all primers of examined genes are described in Supplementary Table 5. Three independent replications were used for each assay and data are presented as means ± S.D. Student's t-test was used to compare the different expression of genes in each comparison and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Sequencing of Splenic Transcriptomes

Next generation sequencing of splenic samples collected at 24-h post-infection produced minimum amount of 11G raw data for each of the 9 libraries. Around 95% of the clean reads had quality scores exceeding the Q 20 value. After removing the

interference data, an average of 71.4% high quality reads was mapped to the chicken reference genome Gallus gallus 4 (see Supplementary Table S1). Among the total of 15,278 detected genes, 1,666 were novel and 11,169 genes were considered in further statistical analysis. Volcano plots, integrating both the *P*-value and fold-change of each transcript were constructed, to show the general scattering of the transcripts and filter the DEGs between the S vs C, R vs C and S vs R, comparisons (Fig. 3-1A, B and C).

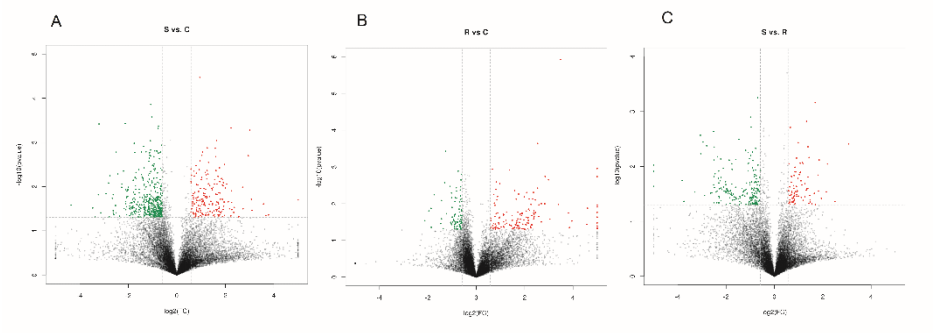

Figure 3-1. Identification of genes differentially expressed among S, R and C chickens during SE infection. Volcano plot showing differentially expressed genes (DEGs) S vs C (A), S vs R (B) and R vs C (C) after *Salmonella* infection. S = challenged-susceptible; R = challenged-resistant; C = Non-challenged controls.

3.2 Differential Expression of mRNAs in Response to Salmonella Infection

The DEGs in spleens of the controls, resistant and susceptible birds were examined. A total of 934 significant DEGs were identified among the S, R and C chickens (Fig. 3-2A). As shown in Fig. 2, 588 genes differed between S and C (176 up- and 412 down-regulated); for R vs C, 234 differed (145 up- and 89 down-regulated); and 265 genes (80 up- and 185 down-regulated) were DEGs between S and R birds (Supplementary Table S2-S4). A total of 32 DEGs were shared in comparisons between S vs C and R vs C birds while 144 DEGs were uniquely expressed in the S vs R comparison. Only 4 DEGs were co-expressed in the 3 contrasts. Hierarchical clustering analysis of the DEGs in each comparison demonstrated that it was appropriate to classify the challenged birds as being resistant or susceptible from their phenotypic evaluation (Fig. 3-2B, 2C and 2D). Several immune-related genes such as *SOCS1*, *CXCR4* and *FOS* were significantly up-regulated ($P < 0.01$) after challenge with *Salmonella* (log2 FC 3.06, 3.69 and 2.50, respectively).

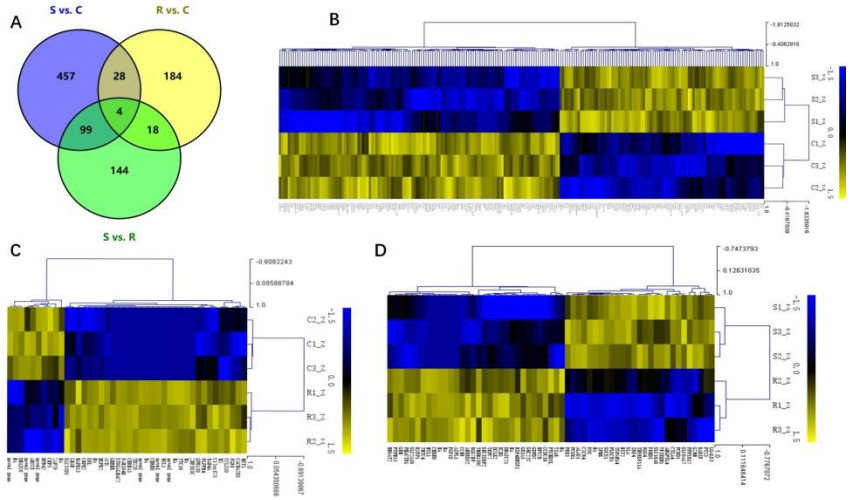

Figure 3-2. Different expression profiles of unique mRNAs in host immune response to SE infection. (A) Venn diagram shows the overlap of DEGs among the 3 groups; numbers are the DEGs in each comparison. (B-D) The heat map of unique DEGs in S vs C, R vs C and S vs R, respectively (FC > 2 & FDR < 0.05). S = challenged-susceptible; R = challenged-resistant; C = Non-challenged controls.

3.3 Significant GO Terms and KEGG Analysis

All the DEGs and unique DEGs in each comparison among S, R and C birds were analyzed using GO and KEGG enrichment. In this study, potential function analysis of all 934 DEGs showed that some significantly enriched GO Terms were mainly involved in channel activity and transmembrane transport but several top immune-related terms were also enriched ($P < 0.05$), including regulation of toll-like receptor signaling pathway (GO:0034121), immune response-activating signal transduction (GO:0002757), B cell receptor signaling pathway (GO:0050853) and regulation of response to stimulus (GO:0048583) (Table 3-1). In addition, Neuroactive ligand-receptor interaction, Cytokine-cytokine receptor interaction and FoxO signaling pathway were significantly changed in response to SE infection ($P < 0.03$, Table 3-2).

Potential functional analyses for host immune responses to SE infection between S and R chickens were further characterized. In the S vs R comparisons, Neuroactive ligand-receptor interaction and Cytokine-cytokine receptor interaction pathway were enriched ($P < 0.05$) (Table 2); the top 3 enriched GO terms were intracellular ligand-gated ion channel activity, ligand-gated channel activity and ligand-gated ion channel activity ($P < 0.01$). For S vs C, 3 pathways were enriched ($P < 0.05$), viz. Cytokine-cytokine receptor interaction, FoxO signaling pathway and Jak-STAT signaling pathway (Table 3-2); and several top

GO terms of the immune response were enriched ($P < 0.01$), including B cell receptor signaling pathway (GO:0050853), regulation of response to stimulus (GO:0048583), and immune response-regulating signaling pathway (GO:0002764). In the R vs C comparisons, Neuroactive ligand-receptor interaction and MAPK signaling pathway were enriched ($P < 0.05$) (Table 3-2); the enriched GO terms ($P < 0.01$) were mainly involved in channel activity, transmembrane transport, cardiac muscle cell proliferation and receptor activity, such as cation channel activity (GO:0005261), transmembrane transporter complex (GO:1902495), signaling receptor activity (GO:0038023) and oxygen transport (GO:0015671). These results indicate that compared to resistant birds, susceptible birds extensively initiate their pathways of immune response, signal transduction, and signal molecules and interaction, presumably in an attempt to resist SE infection.

Table 3-1. Immune-related biological processes identified by gene ontology analysis of differentially expressed genes. All DEGs among C, R and S chickens were used to identify enriched biological functions (*P*-value < 0.05).

term	Description	Count	P-Value
GO:0015267	channel activity	17	0.004
GO:0022803	passive transmembrane transporter activity	17	0.004
GO:0005216	ion channel activity	15	0.011
GO:0038023	signaling receptor activity	27	0.017
GO:0004872	receptor activity	30	0.019
GO:0002224	toll-like receptor signaling pathway	5	0.021
GO:0050853	B cell receptor signaling pathway	3	0.022
GO:0048583	regulation of response to stimulus	59	0.031
GO:0002253	activation of immune response	8	0.036
GO:0050778	positive regulation of immune response	10	0.040
GO:0043065	positive regulation of apoptotic process	12	0.047
GO:0045088	regulation of innate immune response	6	0.048

Table 3-2. Significantly changed immune-related pathways in different contrasts. KEGG pathway analysis was performed with DAVID. *P*-value < 0.05 was considered to be statistically significant pathway.

Class	term	Count	Percents (%)	P-Value	Fold Enrichment
All DEGs	Neuroactive ligand-receptor interaction	27	3.08	0.006	1.73
	Cytokine-cytokine receptor interaction	18	2.05	0.018	1.83
	FoxO signaling pathway	14	1.60	0.025	1.95
	MAPK signaling pathway	21	2.39	0.027	1.65
S vs C	Cytokine-cytokine receptor interaction	16	2.86	0.001	2.53
	FoxO signaling pathway	11	1.96	0.015	2.39
	Jak-STAT signaling pathway	11	1.96	0.012	2.49
R vs C	Neuroactive ligand-receptor interaction	13	6.22	0.000	3.48
	MAPK signaling pathway	8	3.83	0.028	2.64
S vs R	Neuroactive ligand-receptor interaction	10	4.24	0.025	2.31
	Cytokine-cytokine receptor interaction	7	2.97	0.051	2.56

3.3 Quantitative real-time PCR (qPCR) Validation.

The qPCR assays were conducted to validate 16 selected DEGs from RNA-seq: *IL10RB*, *TNFSF10*, *LAMP1*, *ZNF207*, *CCND1*, *GJA1*, *FTH*, *HBBA*, *GAL1*, *CREBBP*, *BRI3BP*, *SOCS1*, *ICOS*, *CTLA4*, *AVD* and *IL8*. Pearson's correlation of the fold-changes between qPCR and RNA-seq was 0.92 (Fig. 3-3). Overall, the RNA-seq results were considered to be reliable and appropriate for further analysis.

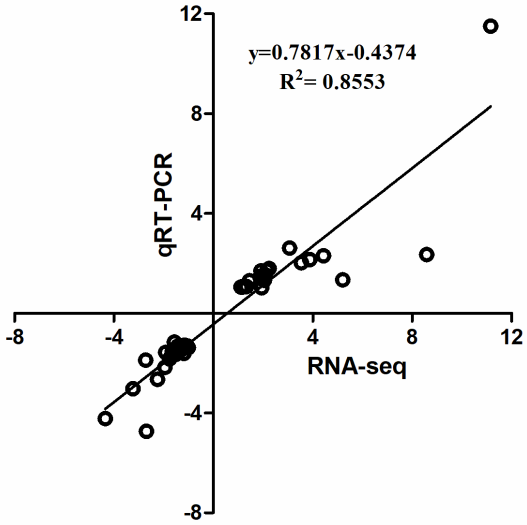

Figure 3-3. Linear regression fitted for Log_2 Fold Change (FC) of selected genes determined via qPCR and RNA-seq. The selected genes in each comparison were used for linear regression analysis. Log_2 FC in RNA-seq equals $2^{-\Delta\Delta Ct}$ in qPCR for each comparison.

4. Discussion

Although there are previous studies focused on the chicken splenic transcriptome following *Salmonella* challenge, the novel experimental design of the current study enabled exposure of the resistance and susceptibility mechanisms of phenotypically different birds in host immune response to *Salmonella* Enteritidis infection. In this research, both the extent of clinical symptoms and the bacterial load in blood were used to assess birds after infection to distinguish resistant from susceptible chickens. And RNA-seq was used here to identify differences splenic mRNAs expression profiles in chicks following SE infection. A total of 934 DEGs were identified among the C, R and S birds. After SE infection, several up-regulated unique DEGs were mainly related to immune function, such as *FOS*, *SOCS1*, *IL18*, *IKBKB*, *CXCR4*, *CTAL4*, *IL10RA*, *IL10RB*, *IL1RAP*, and *AVD*. These findings differ from the splenic results after *Salmonella* infection (Matulova et al., 2012), although they did identify avidin (*AVD*) and immune responsive gene 1 (*IRG1*). The differences may have arisen from genetics or ages of the chickens used, or the bacterium used for challenge (Beaumont et al., 2009; Redmond et al., 2009; Zekarias et al., 2002). In generation of the heatmaps, genes included were largely driven by the S chicks. Comparing the change for each of the 3 groups, they clustered as expected based on earlier contrast comparisons (Fig. 3-2).

It was clear, from the bacterial burden in blood, that septic infection occurred in the challenged birds. In response to systemic infection with *S.* Typhimurium, pro-inflammatory cytokines that are host-produced are critical for the control of bacterial growth but bacterial clearance is dependent on the successful activation of $CD4^+$ T cells, especially in peripheral immune organs (Talbot et al., 2009). Unfortunately, high doses of LPS or *Salmonella* can result in production of excess amounts of pro-inflammatory cytokines, or a "cytokine storm," leading to endotoxin shock or sepsis-related deaths (Clark and Vissel, 2017; Cohen, 2002; Netea et al., 2017). Thus, the potential influence of over-expression of inflammatory cytokines due to hypersensitivity response to SE in susceptible birds was also considered in this study.

After challenge with SE, the significantly changed pathways included Cytokine-cytokine receptor interaction, FoxO signaling pathway, Neuroactive ligand-receptor interaction and MAPK signaling pathway. Consistent with previous studies (Chiang et al., 2008; Li et al., 2017a; Matulova et al., 2012), many immune-related pathways (Cytokine-cytokine receptor interaction, MAPK, and Jak-STAT signaling pathway) have been identified in susceptible chickens following *Salmonella* infection. Importantly, the FoxO signaling pathway is reported here for the first time.

The Forkhead box O (FOXO) is one subfamily of the fork head transcription factor family with important roles in cell fate decisions, including cellular differentiation, apoptosis, cell proliferation, DNA damage and repair and as mediators of oxidative stress (Farhan et al., 2017; Vurusaner et al., 2012). FoxO activity is mainly regulated by the PI3K (phosphoinositide 3-kinase) pathway, whereas FoxO function is negatively "fine-tuned" by protein kinase B (PKB; also known as AKT), casein kinase 1 (CK1) and IκB kinase (IKK) (Peng, 2008). Research shows that FoxO3 gene is strongly considered to regulate lymphoid homeostasis in host immune system (Lin et al., 2004; Lu et al., 2017). For instance, FoxO3a overexpression induces apoptosis in a human leukemia T cell line (Brunet et al., 1999), murine CTLL-2 T cell line (Stahl et al., 2002), murine pre-B cell line Ba/F3 (Dijkers et al., 2002), murine peritoneal macrophages (Senokuchi et al., 2008) and BCG-infected macrophages (Haoues et al., 2014). Deficiency of *FoxO3a* in mice leads to spontaneous, autoreactive helper T cell activation and Th1 and Th2 cytokine production (Peng, 2008), which is required for controlling bacterial growth and clearance following *Salmonella* infection (Chappell et al., 2009; Talbot et al., 2009). In addition, Foxo3a clearly plays critical roles in neutrophil survival, as demonstrated by Foxo3a-deficient mice which are resistant to both peritonitis and arthritis (Jonsson et al., 2005). In the current study, signaling adapter molecules EGFR, IRS1 and PIK3CD of the PI3K pathway, as well as IKBKB gene of IκB kinase, were all significantly reduced in S vs C birds. Of

particular note, the gene *FoxO3* and apoptosis gene *Bcl-6* were significantly up-regulated only in susceptible (S) birds (Fig. 3-4). These results, together with what is known of the immunobiology of avian systemic salmonellosis, indicate that the FoxO signaling pathway plays an important role in response against SE infection. Based on the above analysis, it is hypothesized that the hyperactive *FoxO3* in susceptible chickens might both enhance apoptosis of T, and B lymphocytes and macrophages in the spleen, and constrain production of Th1 and Th2 cytokine, all of which are necessary for immunological clearance during the early stage of *Salmonella* infection.

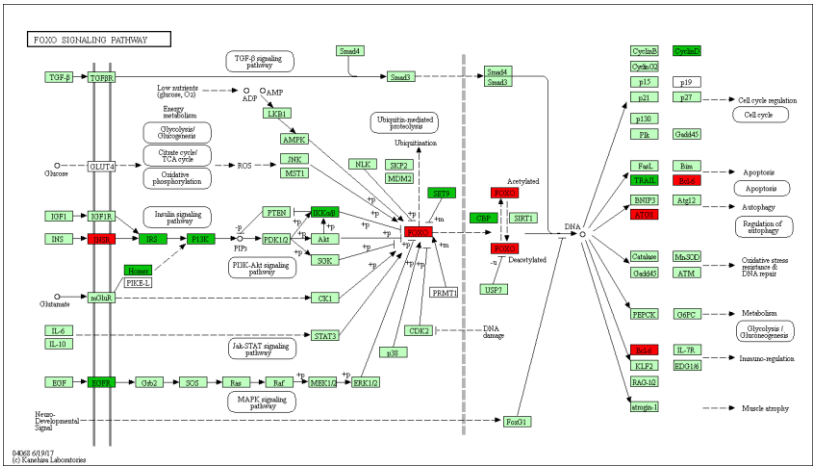

Figure 3-4. FoxO signaling pathway. Red = significantly up-regulated differentially expressed genes. Green = significantly down-regulated differentially expressed genes. Light green represents the genes involved in the pathway.

Many immune-related pathways were significantly induced in S birds (S vs C or S vs R comparisons), including cytokine-cytokine receptor interaction, Jak-STAT, MAPK signaling pathway and neuroactive ligand-receptor interaction (Table 3-2). Interestingly, cytokine-cytokine receptor interaction, MAPK, and Jak-STAT signaling had cross-talk with activating the FoxO signaling pathway (Fig. 3-5). These results suggested that multiple signaling pathway cascades control *Salmonella* invasion and clearance. In addition, increased expression of many genes in these identified pathways, in response to APEC infection, has also been demonstrated (Li et al., 2011; Sandford et al., 2012; Sun et al., 2015, 2016).

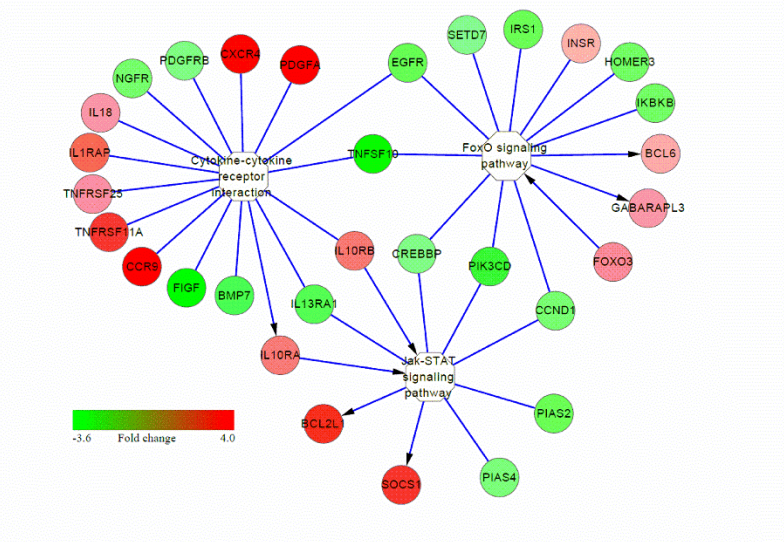

Figure 3-5. Interaction among several signaling pathways involved in susceptibility to *Salmonella* Enteritidis infection. Color column from red to green represents the fold change (FC 4.0 to -3.6) of genes in S vs C or S vs R chickens. Red represents up-regulation and green represents down-regulation; yellow represents the pathway. The arrow represents direct activation.

CXCR4 is expressed in multifarious types of cancer. This cytokine receptor and its ligand are also involved in the recruitment of T cells at the site of the immune or inflammatory reactions (Sallusto and Baggiolini, 2008; Yusuf et al., 2005). TNFRSF11A is a member of the tumor necrosis factor receptor (TNFR) molecular sub-family, also known as receptor activator of nuclear factor κB (RANK). Most commonly, over-expression of *RANK* alone is sufficient to activate the NF-κB pathway (Tracey et al., 2008). Interleukin 10 receptor A and B (*IL10RA*, and *IL10RB*) are expressed in most immune cells, and very low expression levels have been observed on a variety of non-hematopoietic cells (Moore et al., 2001; Yao et al., 2012). Differential expression of *IL10RA* plays an important role in IL10-mediated immune regulation, and activation of monocytes and neutrophils increases mRNA expression, whereas expression levels have been shown to decrease following stimulation of human T cells, B cells and NK cells (Carson et al., 1995; Denning et al., 2000; Jurlander et al., 1997; Yao et al., 2012). IL1 receptor accessory protein (IL1RAP) mediates the response to IL1, IL33, and IL36 and has been shown to regulate the inflammatory response, as well as activation of T lymphocytes and mast cells (Barreyro et al., 2012; Boraschi and Tagliabue, 2013; Dinarello, 2018). High *IL1RAP* expression is associated with poor overall survival in acute myeloid leukemia (AML) patients (Barreyro et al., 2012); although several receptors genes were increased here, only *IL18* genes were significantly up-

regulated. The expression of *IL18* was lower in tumor-associated macrophages cultured with metastatic gastric cancer cell lines (Shen et al., 2012). In the current study, the key genes (*CXCR4*, *TNFRSF11A*, *IL10RAP* and *IL10RB*) in the cytokine-cytokine receptor interaction pathway had increased expression, both in S vs C and S vs R comparisons. While the inflammatory cytokines response is critical for the control of bacterial growth (Cross et al., 1995), excessive cytokines production can lead to endotoxic shock or sepsis-related deaths (Clark and Vissel, 2017; Cohen, 2002; Netea et al., 2017). Overall, these results suggest that susceptible birds showed hypersensitivity to acute SE infections and that the cytokine-cytokine receptor interaction pathway is an important mediator in SE-induced pathogenesis.

The Jak-STAT pathway is needful to ensure T and B cell development (Rawlings et al., 2004). In the present study, various genes involved in the Jak-STAT signaling pathway had increased expression in susceptible birds than in non-infected controls, including *IL10RA*, *IL10RB*, BCL2-like 1 (*BCL2L1*) and suppressor of cytokine signaling 1 (*SOCS1*). BCL2L1 is one the family of Bcl-2 proteins with important roles in the regulation of mitochondrial pathway of apoptosis (Cory and Adams, 2002) and SOCS1 is a negative regulator of LPS-induced macrophage activation (Alvarez et al., 2017; Kinjyo et al., 2002). Interestingly, three overlapping elements were found in Jak-STAT and cytokine-cytokine receptor interaction pathways in susceptible birds, both in S vs C and S vs R comparisons (Fig. 5). These results indicate that susceptible birds extensively activate key pathways of immune response, signal transduction, and signal molecules and interaction in an attempt to resist SE infection, but fail to do so and succumb.

In addition, resistant chicks seem to activate the MAPK signaling in regulating the host response to SE infection. MAPK signaling was shown to be activated in chicks when pathogenic bacteria invaded (Withanage et al., 2004). It was reported that P38 MAPK is very important for B cell development and is a survival mediator for T-cells in human inflamed tissues (Huang et al., 2009). In the current study, 5 up-regulated DE genes (*MAPT*, *MAPK13*, *CACNA2D3*, *CACNG5* and *FGF9*) participated in activating MAPK signaling pathway in resistant birds (Supplementary Table S3). These results are consistent with SE-infected, resistant birds increasing proliferation of T and B lymphocytes in the spleen to achieve protection against *Salmonella*.

5. Conclusion

A total of 934 DEGs were identified in comparisons between the C, R and S birds (R vs C, S vs C and S vs R). Cytokine-cytokine receptor interaction, Jak-STAT and FoxO signaling pathway were activated extensively and there was

cross-talk between them in challenged-susceptible birds. These findings will facilitate the understanding of resistance and susceptibility to SE infection in the earliest phases of the host immune response and provides new approaches to developing strategies for SE prevention and treatment.

Acknowledgements

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4

Identification of miRNAs involved In the host response to *Salmonella* infection

In this Chapter, the aim is to identify the miRNAs and miRNA target genes responsible for host resistance and susceptibility to SE infection using next generation sequencing.

Adapted from Peng Li, Wenlei Fan, Qinghe Li, Jie Wang, Ranran Liu, Nadia Everaert, Jie Liu, Yonghong Zhang, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. 2018. Splenic microRNA expression profiles and integration analyses involved in host responses to *Salmonella* Enteritidis infection in chickens. *Front. Cell. Infect. Microbiol.* 2017, 7:377.

Abstract: To understand the role of miRNAs in regulating genes involved in the host response to *Salmonella* Enteritidis (SE) infection, next generation sequencing was applied to explore the altered splenic expression of microRNAs (miRNAs) and deregulated genes in specific-pathogen-free chickens. Birds were either infected or not (controls, **C**) and those challenged with SE were evaluated 24 h later and separated into 2 groups on the basis of the severity of clinical symptoms and blood load of SE: resistant (**R**, SE challenged-slight clinical symptoms and $< 10^5$ cfu / 10 μL), and susceptible (**S**, SE challenged-severe clinical symptoms and $> 10^7$ cfu / 10 μL). Thirty-two differentially expressed (DE) miRNAs were identified in spleen, including 16 miRNAs between S and C, 13 between R and C, and 13 between S and R. Through integration analysis of DE miRNAs and mRNA, a total of 273 miRNA-target genes were identified. Functional annotation analysis showed that Apoptosis and NOD-like receptor signaling pathway and adaptive immune response were significantly enriched ($P < 0.05$). Interestingly, apoptosis pathway was significantly enriched in S vs C, while NOD-like receptor pathway was enriched in R vs C ($P < 0.05$). Two miRNAs, gga-miR-101-3p and gga-miR-155, in the hub positions of the miRNA-mRNA regulatory network, were identified as candidates potentially associated with SE infection. These 2 miRNAs directly repressed luciferase reporter gene activity via binding to 3'-untranslated regions of immune-related genes *IRF4* and *LRRC59*; over-expressed gga-miR-155 and interference gga-miR-101-3p in chicken HD11 macrophage cells significantly altered expression of their target genes. These findings facilitate better understanding of the mechanisms of host resistance and susceptibility to SE infection in chickens.

Keywords: MicroRNA; *Salmonella* Enteritidis; next generation sequencing; chicken; clinical symptoms; spleen; miRNA-target genes

1. Introduction

Salmonella Enteritidis (SE) is a Gram-negative enteric pathogen, infection with which does not cause significant disease or mortality, but birds can carry the bacteria for several weeks without presenting any clinical signs, thereby constituting an insidious risk for public health (Barrow et al., 2012; Calenge and Beaumont, 2012; Calenge et al., 2010). Although *Salmonella* contamination can be significantly reduced using control measures in poultry, there was a considerable increase in reported *Salmonella* cases in the EU (EFSA and ECDC, 2016) and UK (Inns et al., 2015). SE also tends to be highly resistant to multiple antimicrobials, such as sulfamethoxazole-trimethoprim and nalidixic acid, which has the potential to complicate treatment of animal and human disease (DuPont and Steele, 1987; Goldman, 2004; Kuang et al., 2015). Therefore, to reduce economic losses in poultry production and to protect animal and human health, it is critical to understand the host immune response and mechanisms of resistance against SE infection.

MicroRNAs (miRNAs) have been identified as key regulators of gene expression at the post-transcriptional level. These small RNAs have been demonstrated to have important functions in a variety of biological processes including the cell cycle, differentiation, apoptosis and pathogenesis (Ambros, 2004; Filipowicz et al., 2008; Krol et al., 2010; Yates et al., 2013). There are increasing evidence that the miRNAs play important roles in regulating the innate immune response induced by bacteria (Das et al., 2016; Eulalio et al., 2012; Maudet et al., 2014; Staedel and Darfeuille, 2013). Previous studies have shown that miRNAs, such as miR-146a, miR-155 and Let-7 and their targets are involved in the regulation of immune response to *Salmonella* or lipopolysaccharide infection in mice (O'Neill et al., 2011; Eulalio et al., 2012; Schulte et al., 2011) and swine (Bao et al., 2015; Bao et al., 2014; Yao et al., 2016a; Yao, et al., 2016b). For instance, few proteins (IRAK1, IRAK2 and TRAF6) within TLR signaling have been confirmed as direct targets of miR-146 (O'Neill et al., 2011); signal molecules MyD88, TAB2, SHIP1 and SOCS1 were targets of miR-155 (Eulalio et al., 2012); and cytokines IL-6 and IL-10 are targeted by Let-7 (Staedel and Darfeuille, 2013).

The role of miRNA in response to bacterial infection has also been investigated in chickens. Several miRNAs (gga-miR-125b-5p, gga-miR-34a-5p, gga-miR-1416-5p and gga-miR-166) associated with SE infection were identified recently in laying chicken cecum by next generation sequencing (Wu et al., 2017). A novel splenic miRNA, gga-miR-429, involved in the host response to Avian pathogenic *Escherichia coli* (APEC) was also detected by deep sequencing (Jia et al., 2017). Despite these studies, there is still limited information about the function of miRNAs in the host response and resistance to *Salmonella* infection in chickens.

The spleen, as the body's major blood filter, plays a major role in detecting cell damage during *Salmonella* infection and in the pathogenic mechanisms of *Salmonella*. Further, increasing evidence suggests that the spleen plays a greater role in immune function in avian than in mammalian species, and is responsible for an immediate innate reaction after recognizing pathogens by filtering antigens from the blood (Smith and Hunt, 2004; Tiron and Vasilescu, 2008). Assessing changes in the expression of miRNAs and their targets in spleen on a genome-wide scale, therefore, could provide more comprehensive insight into the immune response to bacterial infection. The objectives of the present study were to identify the miRNAs and miRNA-regulated genes responsible for host resistance and susceptibility to SE infection using next generation sequencing on spleens from 3 groups of chickens: Controls (C, non-challenged, no detected SE in blood at 24 h, Resistant (R, SE-challenged, slight clinical symptoms, $< 10^5$ cfu / 10 μL SE in blood), and Susceptible (S, SE-challenged, severe clinical symptoms, $> 10^7$ cfu / 10 μL SE in blood) chickens. Subsequently, based on combined analysis of expression profiles of miRNA and potential target mRNA, the functional analysis and candidate miRNAs involved in the host response to SE infection were further characterized with the goal of better understanding the mechanisms of resistance and susceptibility to *Salmonella*.

2. Materials and methods

2.1 Ethics statement

All of the animal experiments were conducted in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). Animal experiments were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China). Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS (approval number: IASCAAS-AE20140615).

2.2 Animals and sample collection

Specific-pathogen-free White Leghorn chickens were supplied by the Beijing Laboratory Animal Research Center (BLARC, Beijing, China) and were treated as described in previous studies (Gou et al., 2012; P. Li et al., 2010). In brief, the SPF chickens were raised in climate-controlled, fully enclosed isolation facilities at the experimental center of China Agriculture University (Beijing, China) under identical management conditions. At 3 d of age, a total of 150 SPF chickens were orally challenged with 1 ml PBS containing 10^8 cfu of *S.* Enteritidis (50041) and another

75 birds received 1 ml PBS as controls. Blood samples from each of 30 challenged and 15 control chickens were taken at 24 h post infection and birds were killed and the spleens were dissected, snap frozen and held at -80°C. Bacterial burden (expressed as cfu/10 µL blood) was determined indirectly by serovar-specific quantitative real-time PCR (qPCR), and along with clinical severity, was used to evaluate the resistance/susceptibility to SE challenge, as described in previous studies (Deng et al., 2008; Gou et al., 2012). 10 µL EDTA-anticoagulated blood was used for DNA extraction using MiniBEST Whole Blood Genomic DNA Extraction Kit (Takara, Code No. 9781) according to the manufacturer's instructions. Amplification was carried out in a total 25 µL reaction mixture, containing 0.6 µL of each primer (10 µM), 0.75 µL of dNTPs (10 mM), 1.25 U of ExTaq DNA Polymerase (Takara), 5.5 µL of 5 × PCR buffer (Mg^{2+}), 0.8 µL of TaqMan probe (5 µM), and 2 µL of template, with deionized water to 25 µL. Each PCR consisted of a 5 min hot start at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and a fluorescence read step. The probe (5'-FAM-TGCAGCGAGCATGTTCTGGAAAGC-TAMRA-3') and primers set (forward primer, 5'-TCCCTGAATCTGAGAAAGAAAAACTC-3'; reverse primer, 5'-TTGATGTGGTTGGTTCGTCACT-3') were designed from the *SdfI* gene (GenBank Accession No. AF370707.1), as described in Gou et al (2012). The qPCR assay was calibrated by relating threshold cycle (Ct) values to cfu, as determined by enumeration after plating serial dilutions of *S.* Enteritidis and standard culture.

In this study, the clinical symptoms and bacterial load of SE at 24 h after challenge were used together to discriminate susceptible (S, SE-challenged, slight clinical symptoms and > 10^7 cfu/10 µL blood) from resistant (R, SE-challenged, severe clinical symptoms and < 10^5 cfu/10 µL blood) birds. No SE was detected in the Controls (C). Total splenic RNA was extracted from 3 birds in each of the 3 groups, S, R and C, using miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA was quantified using the NanoDrop ND-2000 spectrophotometer (NanoDrop Products, Wilmington, DE) and purity was assessed by Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA) with RNA Integrity Number (RIN) number > 7.0. Total RNA was stored at -80°C until used.

2.3 Screening the differentially expressed miRNAs

Total RNA of each sample (approximately 1 µg) was used to prepare the miRNA sequencing library, which included the following steps: 1) 3'-adapter ligation with T4 RNA ligase 2 (truncated); 2) 5'-adapter ligation with T4 RNA ligase; 3) cDNA synthesis with an RT primer; 4) PCR amplification; and 5) extraction and purification of 120-140 bp PCR amplified fragments (corresponding to ~15-25 nt

small RNAs) from polyacrylamide gels. An Agilent 2100 Bioanalyzer quantified the libraries, after which the samples were diluted to a final concentration of 8 pM and cluster generation was performed on the Illumina using TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA), following the manufacturer's instructions. The 9 miRNA libraries were constructed and single-end sequenced (36 bp) on an Illumina Hiseq 2500 at the LC-BIO (Hangzhou, China) following the vendor's recommended protocol. The raw data of each sample was not less than 10M reads. The raw reads were subjected to the Illumina Pipeline filter (Solexa v0.3), and then the dataset was further processed with ACGT101-miRv4.2 (LC Sciences, Houston, TX) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, the 18-25 nt length unique sequences were BLASTed to chicken precursors in miRBase 20.0 (Kozomara and Griffiths-Jones, 2014) (http://www.mirbase.org/) to detect known miRNAs and novel 3p- and 5p- derived miRNAs. One mismatch inside the sequence and length variation at both 3' and 5' ends were allowed in the alignments. The unique sequences were mapped to chicken mature miRNAs in hairpin arms recognized as known miRNAs, and mapped to the other arm of known chicken precursor hairpins opposite to the annotated mature miRNA-containing arm considered to be novel 5p- or 3p-derived miRNAs. The remaining sequences were mapped to other selected species in miRBase 20.0 by BLAST search, and the mapped pre-miRNAs were further BLASTed against the chicken genomes to identify their genomic positions. The aforementioned miRNAs were considered to be known miRNAs. To identify the novel predicted miRNAs, the unmapped sequences were BLASTed against the chicken genome database, and the hairpin RNA structures comprising sequences were identified using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Modified reads per million (RPM) reads was used to quantify the normalized reads, the formula was: Normalized Expression (NE) = Actual miRNA count/Total count of clean reads. MicroRNAs were regarded as being differentially expressed (DE) based on normalized deep-sequencing levels (with the exclusion of 3 RPM) in S, R and C groups, respectively. The DE miRNAs based on normalized counts were analyzed using Student t-tests (Huang et al., 2015; Li et al., 2016) according to the experimental design and the significance threshold was set as $P < 0.05$. The normalized read counts of some miRNAs were set to be 0.01 for further calculation if they had no reads in the library.

2.4 Differentially expressed analysis of mRNA

Nine cDNA libraries were also constructed from splenic RNA (1 μg) of these same birds and sequenced by LC-BIO (Hangzhou, China) on an Illumina HiSeq 2500 platform and 100 bp paired-end reads were generated. The raw reads were first processed through FastQC to obtain the clean data, by removing the reads that

contain sequencing adapter contaminations or poly-N and the low quality reads, Q values for which were < 20. Some potential residual ribosome RNA data were also removed from the remaining data by alignment. Clean reads were then mapped to the Gallus gallus database using TopHat (Trapnell et al., 2009), and the mapped reads were assembled de novo using Cufflinks (Trapnell et al., 2010). Expression levels of mRNAs were quantified as fragments per kilobase of exon per million mapped reads (FPKM) using the Cufflinks package (Trapnell et al., 2010). Analysis of DE genes between the 3 groups of chickens was performed using the Cuffdiff with a $P < 0.05$ and $|\log_2 \text{fold change}| > 0.58$.

2.5 Prediction of DE miRNA targets, Gene Ontology (GO) and KEGG pathway analysis

Only target DE genes that were predicted by both TargetScan 6.2 and miRanda 3.3 for all of the DE miRNAs were considered further. Gene Ontology (GO) and KEGG pathway enrichment of target DE genes were analyzed by DAVID 6.8 (http://david.abcc.ncifcrf.gov/), which is based upon a Fisher Exact statistic methodology similar to that previously described (Huang et al., 2009). GO and KEGG results were filtered using *P*-value < 0.05.

2.6 Correlation analysis of miRNA and mRNA

In order to build the miRNA-mRNA interaction network, the following method was used, as described in previous studies (Bao et al., 2015): A target gene was identified by the direction of change in a pairwise comparison, for example S to C, being the reverse of changes in the miRNAs. The miRNA-mRNAs interaction network was constructed using Cytoscape v2.8.3 software (http://www.cytoscape.org/).

2.7 miRNA target validation

The pmiR-RB-Report™ (RiboBio, Guangzhou, China) including double luciferase reporter genes was used to test and validate the target sites for gga-miR-155 and gga-miR-101-3p. The 3′ UTR of *IRF4* and *LRRC59* containing gga-miR-101-3p and gga-miR-155 binding sites were amplified from chicken genomic DNA. The primers for PCR are provided, as follows: *IRF4*: GGCGGCTCGAGGATCCTCAGAATAAGTGTT (forward) and AATGCGGCC-GCGTTAGAAGTCCCTAGAAAA (reverse); and *LRRC59*: GGCGGCTCGAGA-TGCTACAGCAGAACTCGC (forward) and AATGCGGCCGCCAGACAAATT-GATGCGAAA (reverse). All PCR products were cloned into the pmiR-Repor Vector using Xhol and NotI restriction enzymes. Luciferase reporter experiments were performed in 293T (human embryonic kidney) cells, obtained from ATCC.

Cells were seeded in 96-well plates at a density of 5×10^4 cells/well and cultured under routine conditions with 10% fetal bovine serum. When the cells reached 70% to 80% confluence, pmiR-3' UTR (100 ng) was co-transfected with 50 nM of a negative control or a gga-miR-101-3p mimic (GenePharma, Shanghai, China) using 0.30 μL of FugeneHD (Promega, Madison, WI) according to the manufacturer's instructions. The relative luciferase activity was measured 48 h after transfection by the Dual-Glo Luciferase Assay System (Promega).

2.8 Over-expressed miR-155 and interference miR-101 in vitro

To further validate the biological function of gga-miR-155 and gga-miR-101-3p in a chicken macrophage-like line HD11, 100 μM mimic (gga-miR-155), inhibitor (gga-miR-101-3p) and control oligos (gga-miR-NC) were transfected into HD11 cells using 12-well plates and TransIT®-2020 (Mirus Bio, Madison, WI) per the manufacturer's instructions. HD11 cells were grown at 37°C with 5% CO_2 in RPMI-1640 medium that contained 10 mM HEPES, 1 mM sodium pyruvate, 1% glutamine, 1% MEM NEAA, 10% fetal bovine serum and 5% chicken serum (all reagents from Gibco). After 36-h transfection, the cells were harvested using MiniBEST Universal RNA (Takara, Code No. 9767) to extract the total RNA. To evaluate the effect of miRNAs on the abundance of inflammatory genes, the transfected HD11 cells were stimulated with LPS (*Escherichia coli* 055: B5, Sigma). First the cells were transfected with miRNA control (50 nM), miRNA inhibitor control (100 nM), miRNA-155/101 (50 nM) and miRNA-155/101 inhibitor (100 nM) for 24 h, then stimulated with LPS (1μg / mL) for 6 h in 24-well plates. RNA collection and extraction is the same as above.

2.9 Quantitative real-time PCR Analysis

To validate and characterize the DE miRNA and DE transcripts identified via high-throughput sequencing, qPCR analyses were performed in an ABI 7500 Detection System (Applied Biosystems, Foster, CA). The miScript SYBR Green PCR kit (Qiagen, Valencia, CA) and PCR Master Mix (SYBR Green) Kit (Toyobo, Osaka, Japan) were used in qPCR to determine the abundance of mRNAs and miRNAs, using *β-actin* and *U6* genes as reference genes, respectively. The relative mRNA and miRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three independent replications were used for each assay and data are presented as means ± S.D.

3. Results

3.1 miRNA profiles in the spleen of chickens

An average of 6,348,747 high quality clean reads per miRNA sample, which represented 359,232 unique reads in the range of 18-26 nt in the 9 libraries were obtained from splenic samples via next generation sequencing (Supplementary Table S1). These high-quality reads were mapped to chicken precursors in miRBase to identify known and novel miRNAs for further analysis. Low levels of large fragments, such as mRNA and rRNA, were also found, which indicated the high-quality and minimal degradation of RNA samples in the present study. For all 9 samples, the distribution of the small RNA sequence length was mainly concentrated at 22 nt, followed by 23 and 21 nt (Fig. 4-1A), which is consistent with the typical size range for Dicer-derived products and in agreement with most of the previous reports from other animal species.

A total of 2238 miRNAs, classified into 5 categories (Supplementary Table S2), were detected via BLAST in miRBase. After removing the less expressed miRNAs, i.e., the expression levels were less than 3 after the normalization of dataset (in at most 3 samples), 744 miRNAs were identified including 439 known chicken miRNAs, and 62 potentially novel miRNAs (defined as PC-3p or PC-5p) in chicken spleen after oral challenge with SE (Supplementary Table S2).

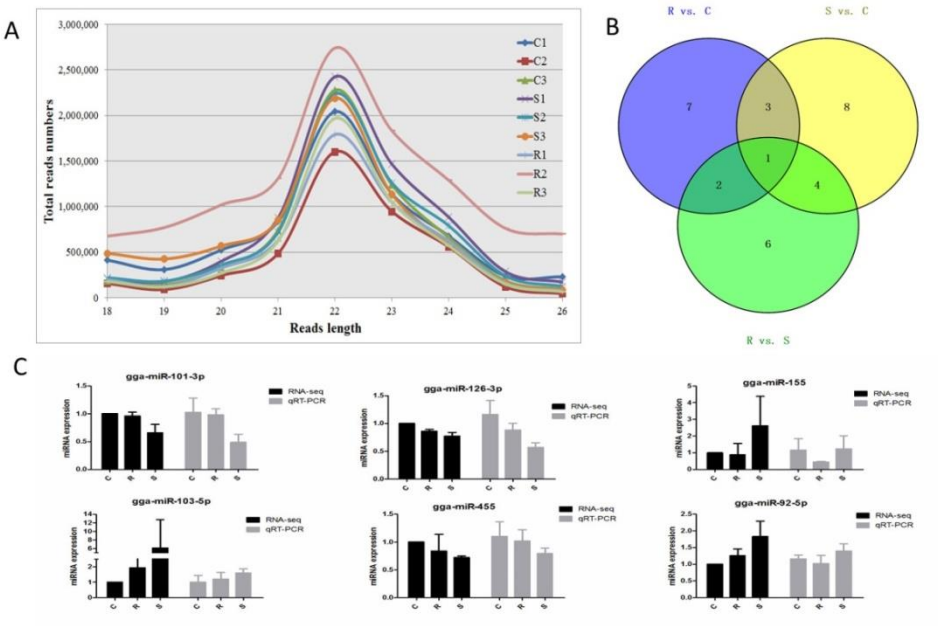

Figure 4-1. Different expression profiles of miRNAs among C, R and S chickens. (A) Size distribution of sequenced small RNA reads. (B) Venn diagram demonstrates the overlap of differentially expressed (DE) miRNAs among the 3 groups; numbers are the DE

miRNAs in each comparison. (C) Correspondence of miRNAs obtained by high-throughput sequencing and qPCR. C = Controls; R = Resistant (cfu < 10^5); S = Susceptible (cfu > 10^7).

3.2 Differential expression of miRNAs

A total of 32 miRNAs exhibited significantly different expression (DE) among the C, R and S groups. The results showed that, for S vs C 16 DE miRNAs (7 up- and 9 down-regulated); for R vs C 13 DE miRNAs were found (4 up- and 9 down-regulated) and 13 were found in the R vs S comparison (10 up- and 3 down-regulated) (Fig. 4-1B and Table 4-1). To validate the expression profiles from sequencing, 6 miRNAs were also examined by q-PCR (Fig. 4-1C). Except for gga-miR-92-5p with a slight difference in the R group, the expression patterns of gga-miR-101-3p, gga-miR-126-3p, gga-miR-155, gga-miR-103-5p, and gga-miR-455 were comparable by both methods. The expression profiles from the deep sequencing were therefore considered as being reliable and appropriate for further analysis.

The differences in splenic expression between the controls, resistant and susceptible birds were examined. Four miRNAs were significantly differently expressed in both S vs C and R vs C, and 5 in both S vs C and S vs R, as well as 3 in both R vs C and R vs S (Fig. 4-1B, Table 4-1). Only 1 miRNA (gga-miR-1677) was significantly differently expressed in all 3 groups of birds (Fig. 1B). Several miRNAs previously reported to be involved in immune responses such as miR-155, miR-9, miR-30, miR-126 and miR-29 families were identified. Also identified here were several new candidate miRNAs associated with SE infection, such as gga-miR-29c-5p (up-regulated, $P = 0.01$) and gga-miR-137-3p (down-regulated, $P = 0.009$).

Table 4-1. Differential expression profile of splenic miRNAs among birds responding differently to SE infection. Three birds in each of the 3 groups were normalized to obtain the expression of transcripts per million using total clean reads count (FKPM) in this study. The *P*-value < 0.05 among C, R and S were considered to be the differentially expressed miRNAs.

miR_name	Control group			Susceptible group			Resistant group			Fold change		
	C1	C2	C3	S1	S2	S3	R1	R2	R3	S vs. C	R vs. C	S vs. R
gga-miR-30d	179,038	261,752	189,247	219,678	229,144	199,988	187,155	180,891	177,550	1.03	0.87	1.19
gga-miR-126-3p	108,338	110,264	129,549	90,066	85,231	90,663	94,410	98,790	107,131	0.76	0.86	0.89
gga-miR-101-3p	45,446	47,287	43,332	38,096	33,688	43,994	41,802	39,141	41,483	0.85	0.9	0.95
gga-miR-130b-3p	20,879	21,193	20,467	19,025	18,282	19,224	16,414	18,857	19,163	0.9	0.87	1.04
gga-miR-155	9,447	14,579	6,040	15,874	22,363	28,150	6,580	7,062	10,569	2.21	0.81	2.74
gga-miR-219b	7,612	9,004	6,773	8,128	7,685	7,064	6,775	6,554	6,625	0.98	0.85	1.15
gga-miR-455-5p	7,149	7,996	8,713	5,330	5,755	6,035	7,359	4,995	6,368	0.72	0.78	0.91
gga-miR-140-5p	3,053	3,135	4,058	2,908	2,964	2,592	3,344	3,602	3,629	0.83	1.03	0.8
gga-miR-181a-3p	2,214	2,963	1,926	3,352	3,973	3,186	2,460	2,659	2,985	1.48	1.14	1.3
gga-miR-1677-3p	1,904	1,900	1,955	1,757	1,862	1,804	1,702	1,588	1,424	0.94	0.82	1.15
gga-miR-1451-3p	233	225	271	160	279	241	197	155	194	0.93	0.75	1.25
gga-miR-137-3p	139	160	154	95	96	65	79	132	100	0.57	0.69	0.82
gga-miR-92-5p	137	192	120	271	253	262	158	219	178	1.75	1.24	1.42
gga-miR-100-3p	110	143	99	206	152	185	108	178	144	1.54	1.23	1.26
gga-miR-1781-3p	106	109	104	107	106	115	95	81	90	1.03	0.84	1.23
gga-miR-9-3p	77	110	86	61	25	45	151	28	39	0.48	0.8	0.6
gga-miR-1769-3p	61	22	40	59	114	129	76	91	73	2.46	1.97	1.26
gga-miR-3539	54	61	46	41	61	51	38	41	34	0.95	0.71	1.35

Table 4-1. (continued)

miR_name	Control group			Susceptible group			Resistant group			Fold change		
	C1	C2	C3	S1	S2	S3	R1	R2	R3	S vs. C	R vs. C	S vs. R
gga-miR-1306-5p	49	55	54	77	63	77	65	81	71	1.37	1.38	1
gga-miR-490-5p	44	30	38	25	1	11	56	20	37	0.33	1.01	0.33
gga-miR-1651-3p	44	47	39	48	44	56	41	37	34	1.14	0.87	1.32
gga-miR-1712-3p	29	38	30	33	37	31	19	25	28	1.04	0.75	1.4
gga-miR-6583-5p	19	20	11	2	4	11	8	11	23	0.34	0.85	0.4
gga-miR-1458	13	11	5	20	32	30	22	30	10	2.83	2.24	1.32
gga-mir-1662-p3	11	9	12	8	6	5	14	17	6	0.59	1.17	0.51
gga-miR-29c-5p	8	12	12	109	13	7	21	20	17	4.03	1.94	2.22
gga-miR-6701-3p	7	8	6	11	7	1	2	4	5	0.9	0.57	1.73
gga-miR-7460-3p	7	8	5	6	5	7	3	1	3	0.9	0.4	2.57
gga-miR-6575-5p	5	3	3	3	5	1	6	7	8	0.82	1.98	0.43
gga-miR-103-5p	2	1	5	8	9	5	1	3	5	2.75	1.47	2.44
gga-miR-1798-3p	1	4	1	11	2	1	13	15	16	2.33	7.72	0.32

3.3 Differential expression of miRNA-targeted genes

In order to validate the roles of DE miRNAs in affecting expression of their target genes, mRNA in the same samples was also profiled by sequencing. Based on both TargetScan and miRanda systems, a total of 273 DE genes can be targeted by the 32 DE miRNAs in the 3 groups. As shown in Fig. 4-2A, 148 (S vs C), 40 (R vs C) and 85 (R vs S) DE miRNA-targeted genes were identified with fold change (FC) >1.50 or < 0.67 and $P < 0.05$ (Supplementary Table S5). The heat map and hierarchical clustering demonstrated distinct profiles of the unique miRNA-targeted genes in the 3 comparisons (Fig. 4-2B, 2C and 2D).

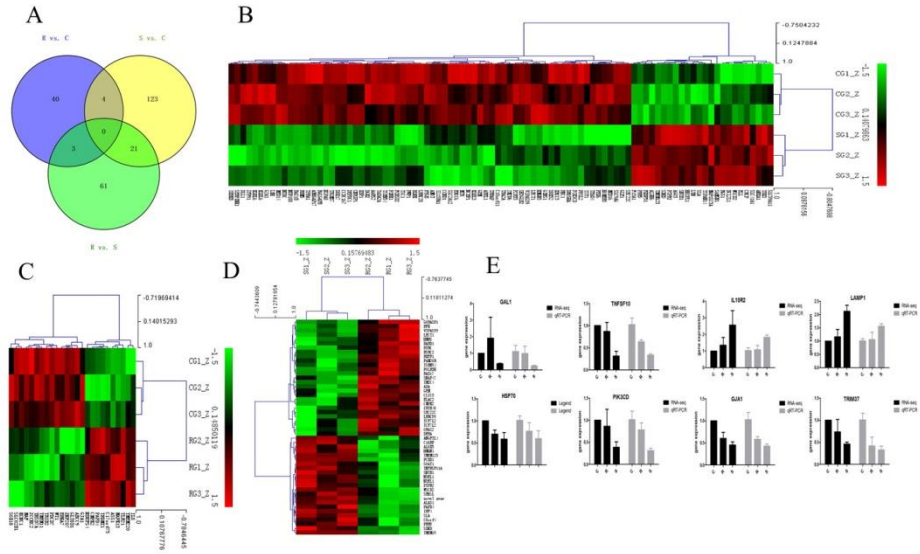

Figure 4-2. Differential expression of miRNA-targeted genes in response to SE infection. (A) Venn diagram demonstrates the overlap of targeted genes for the DE miRNAs among the 3 groups of chickens. Numbers in each section indicate the numbers of differently expressed miRNAs in the comparison. (B-D) The heat map of unique targets of DE miRNAs in S vs C, R vs C and R vs S, respectively. (E) Correspondence of the targeted genes for the DE miRNAs by high-throughput sequencing and qPCR. Data for each method were from the same samples of splenic tissues (C, R and S chickens); TargetScan 6.2 and miRanda 3.3 were used to predict the miRNA targets and only targets predicted by both methods were used for further analysis. The heat map and clustering was constructed by Multi Experiment Viewer v4.8 using Row Z-Score (Murie et al., 2014) [(ΔΔCt–means)/SD] (Supplementary Table S7). In the figures, red represents up-regulation, green shows down-regulation, and black is no change.

Several immune-related genes were found to be significantly DE in spleen after

challenge with SE. For example, the expression of *IL8*, *CXCR4* and *IRF4* were significantly up-regulated following SE challenge (FC 5.21, 3.69 and 2.02, respectively). To validate the expression profiles from sequencing, transcript abundances of 8 genes were measured by qPCR (Fig. 4-2E); overall, there was good concordance between the two methods.

3.4 Potential function analysis of DE miRNA targets

The ultimate function of miRNAs is at the level of the activity of target genes. In this study, functional annotation and pathway enrichment analysis of 273 target DE genes were performed using Gene Ontology (GO) and KEGG. Potential function analysis of these genes showed that 2 immune-related KEGG pathways and 1 biological process were significantly enriched ($P < 0.05$), including Apoptosis, NOD-like receptor signaling pathway, and adaptive immune response (GO:0002250) (Table 4-2). The present results suggest that the changed miRNAs may regulate these immune-related targets in chicken spleen during SE infection.

Table 4-2. Functional annotation and pathway enrichment analysis of all target genes were performed using GO and KEGG. The potential targets of 32 differentially expressed miRNAs among C, R and S chickens were used to identify enriched biological functions (*P*-value < 0.05). Only biological processes are listed.

Term	Description	Count	Percent%）	*P*-value
gga04210	Apoptosis	5	2.1	1.50E-02
gga00562	Inositol phosphate metabolism	5	2.1	3.41E-02
gga04621	NOD-like receptor signaling pathway	4	1.7	3.74E-02
gga04630	Jak-STAT signaling pathway	6	2.6	5.66E-02
GO:0018149	Peptide cross-linking	4	1.7	1.54E-03
GO:0002250	Adaptive immune response	4	1.7	2.38E-02
GO:0001525	Angiogenesis	6	2.6	3.77E-02
GO:0000320	Re-entry into mitotic cell cycle	2	0.9	3.98E-02

Potential functional analyses for host immune responses to SE infection between R and S chickens were further characterized, based on the target genes of significant DE miRNAs between these 2 groups and the controls. For S vs. C, 4 pathways were enriched ($P < 0.05$), viz. Apoptosis, Spliceosome, mTOR signaling pathway, Insulin signaling and Jak-STAT signaling pathway; 2 biological processes were significantly enriched ($P < 0.05$); regulation of inflammatory response and heart looping. In the R vs. C comparisons, NOD-like receptor signaling pathway was significantly enriched ($P < 0.05$); defense response to bacterium (GO:0042742), immune-related biological processes, was enriched but not significantly ($P = 0.06$).

Except for regulation of proteasomal protein catabolic process (GO:0061136) being enriched ($P < 0.05$), no pathways were found when R was compared with S (Table 4-3). These results are consistent with the susceptible birds being more likely to exhibit apoptosis due to an inflammatory response, while the resistant birds showed more of an innate immune response to SE infection.

Table 4-3. GO and KEGG enrichment of unique miRNA target genes were analyzed between S vs. C, R vs. C, and S vs. R. The results were filtered using *P*-value < 0.05.

Class	term	Count	%	*P*-Value
	Apoptosis	4	4.1	6.00E-03
	Spliceosome	4	4.1	3.81E-02
S vs. C	Jak-STAT signaling pathway	4	4.1	4.70E-02
	Insulin signaling pathway	4	4.1	4.70E-02
	mTOR signaling pathway	3	3.1	4.70E-02
	GO: regulation of inflammatory response	3	3.1	2.80E-02
R vs. C	NOD-like receptor signaling pathway	2	14.3	4.60E-02
	GO: defense response to bacterium	2	8.3	6.00E-02
S vs. R	GO: regulation of proteasomal protein catabolic process	2	4.1	3.20E-02

3.5 miRNA-mRNA regulatory relationships

Most descriptions of miRNA function have focused on their roles as post-transcriptional regulators of target mRNAs. Based on the putative miRNA-mRNA regulatory pairs, it was found that 91 SE-related genes can be targeted by 29 of the 32 DE miRNAs. The potentially important interaction networks for immune-related miRNA-mRNA pairs are shown in Fig. 4-3. The relative expression of innate/inflammatory marker genes such as *PIK3CD* was significantly up-regulated following SE infection. Some mRNAs are highly connected and regulated by multiple miRNAs. For example, *CXCR4* is involved in cytokine-cytokine receptor interaction and was identified as a potential target of gga-miR-155 and gga-miR-9-3p. *IFR4* was predicted to be regulated by gga-miR-30d and gga-miR-101-3p. *LRRC59* was predicted as a potential target of gga-miR-103-5p and gga-miR-155. One *Salmonella*-regulated miRNA of particular interest identified through the present study is gga-miR-101-3p. Although the expression levels of miR-101-3p were relatively moderate, it is highly connected (> 8 SE-related target genes) within the miRNA-mRNA network. These have not been previously reported to be

associated with *Salmonella* infection, and are predicted here to regulate several immune-related genes.

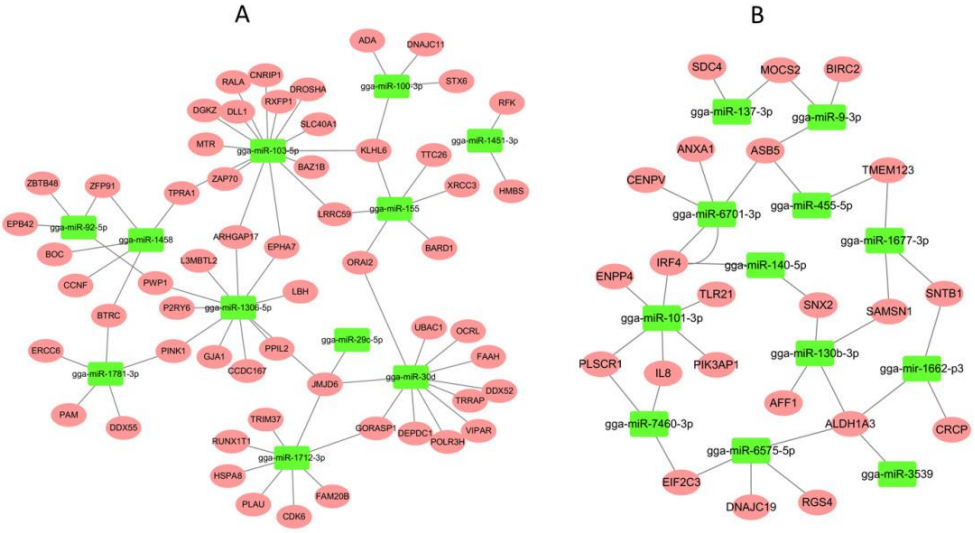

Figure 4-3. miRNA-mRNA interactions in spleen associated with SE infection. (A) miRNA-mRNA network among up-regulated miRNAs and down-regulated mRNAs (B) miRNA-mRNA network among down-regulated miRNAs and up-regulated mRNAs.

3.6 Validations of miRNA-mRNA interactions in vitro

The luciferase reporter gene system was used to validate the above-stated predicted interactions. The 3' UTRs of *IRF4* and *LRRC59* were cloned into luciferase reporter plasmids to test gga-miR-101-3p and gga-miR-155 functions in vitro. Transfection with a gga-miR-101-3p mimic resulted in significant ($P < 0.01$) reduction in relative luciferase activity for *IRF4* plasmids (Fig. 4-4), compared with negative control miRNA (random miRNA sequence) and a no-insert control. Similarly, transfections with mimics resulted in significant ($P < 0.05$) reduction in relative luciferase activity for *LRRC59* (Fig. 4-5) compared with the negative miRNA and no-insert controls. These results indicate that similar responses are likely to be happening in the host during SE infection, that is, the down-regulation of gga-miR-101-3p may result in increased expression of *IRF4* during *Salmonella* infection, and up-regulation of gga-miR-155 may inhibit expression of *LRRC59*.

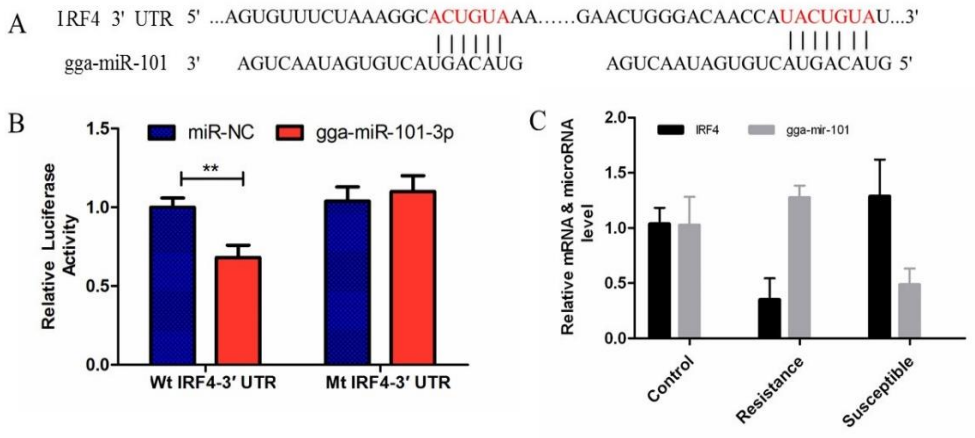

Figure 4-4. Regulation of *IRF4* by gga-miR-101-3p. (A) Predicted gga-miR-101-3p binding sites at distinct positions in *IRF4*; nucleotides of the gga-miR-101-3p seed region are in red. (B) Luciferase activity in 293T cells transfected with miRNA mimics and plasmids carrying the 3'UTR of *IRF4*. NC miRNA = negative control miRNA. (C) Expression change of *IRF4* and gga-miR-101-3p after infection.

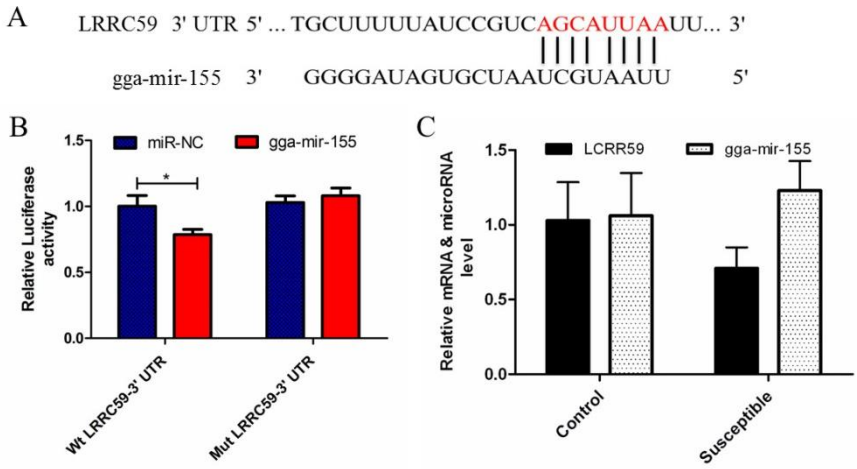

Figure 4-5. Regulation of *LRRC59* by gga-miR-155. (A) Predicted gga-miR-155 binding sites at distinct positions in *LRRC59*; nucleotides of the gga-miR-155 seed region are in red. (B) Luciferase activity in 293T cells transfected with miRNA mimics and plasmids carrying the 3'UTR of *LRRC59*. NC miRNA = negative control miRNA. (C) Expression change of *LRRC59* and gga-miR-155 after infection.

3.7 Validations of miRNAs function in chicken macrophage cell

After 36-h treatment with mimic, elevating gga-miR-155 significantly repressed the mRNA expression levels of *LRRC59* compared to the miR-NC and negative controls ($P < 0.05$); In contrast, after 36-h treatment with gga-miR-101-3p inhibitor, the mRNA expression levels of *IRF4* were significantly increased ($P < 0.05$) compared to the controls (Fig. 6). In order to address the effect of miR-155 and miR-101 on the induction of pro-inflammatory cytokines in response to LPS, the expression levels of *IL-6* and *TNF-α* were measured in a macrophage inflammatory response model. The results showed that miR-155 overexpression markedly decreased the expression of *IL-6* and *TNF-α* compared with control miRNA or miR-155 inhibitor (Figure 4-7A; $P < 0.01$), while miR-101 knockdown significantly decreased the expression of *IL-6* and *TNF-α* compared with control miRNA inhibitor (Figure 4-7B; $P < 0.05$). These data demonstrate that gga-miR-155 and gga-miR-101 could regulate the production of pro-inflammatory cytokines, *IL-6* and *TNF-a*, which may play a negative role in response to LPS stimulation in chickens.

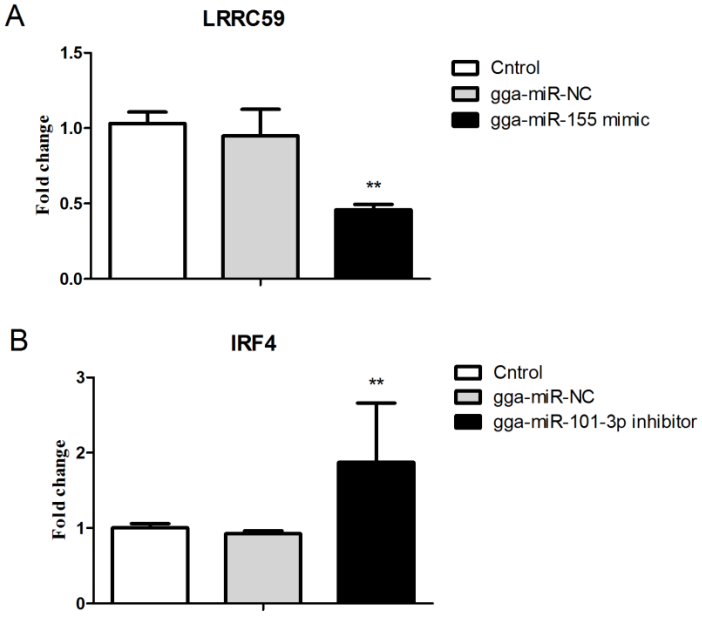

Figure 4-6. Validations of biological function of gga-miR-155 and gga-miR-101-3p in chicken HD11 macrophages. (A) gga-miR-155 mimic significantly repressed the mRNA expression of *LRRC59*. (B) gga-miR-101-3p inhibitor significantly promoted mRNA expression of *IRF4*. The fold-change values were calculated using the comparative $2^{-\Delta\Delta CT}$.

The *P*-values are indicated with asterisks when lower than 0.05 (*) or 0.01 (**) when compared to control (non-transfected) and NC (gga-miR-NC).

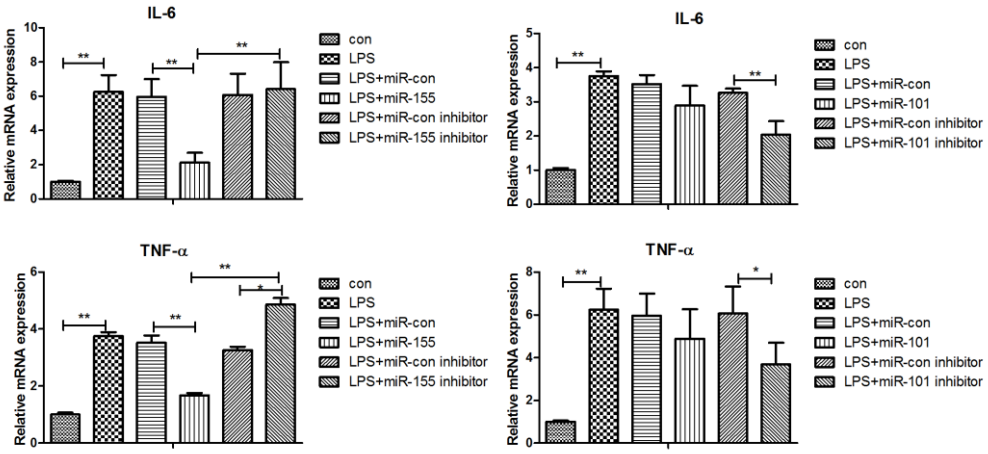

Figure 4-7. Gga-miR-155 and gga-miR-101-3p regulates the production of proinflammatory cytokines induced by LPS. mRNA expression of *IL-6* and *TNF-α* in chicken HD11 6 h after LPS treatment, or 24 h post transfection with miRNA control (50 nM), miRNA inhibitor control (100 nM), miRNA-155/101 (50 nM) and miRNA-155/101 inhibitor (100 nM) then the cells were stimulated with LPS (1μg / mL) for 6 h. The expression levels of the genes were analyzed by qPCR. Data are presented as the mean ± SE from three independent experiments performed in triplicate. The *P*-values are indicated with asterisks when lower than 0.05 (*) or 0.01 (**) when compared to control.

4. Discussion

MiRNAs are important regulators of innate and adaptive immunity (O'Neill et al., 2011; Olivieri et al., 2013; Sonkoly et al., 2008) but their specific roles in regulating the responses to *Salmonella* infection in chicken are incompletely understood. It is necessary, therefore, to identify and characterize the critical miRNAs in the chicken immune response to *Salmonella* with the aim of understanding pathogenesis, improving animal welfare, reducing losses in poultry production and in keeping food safe. Here, next generation sequencing was used to detect differences in splenic expression profiles of miRNAs in chickens challenged with SE. A total of 439 known and 62 potentially novel miRNAs were detected, including those expressed at low levels such as gga-miR-7460 (normalized average 7 and 2 reads for C and R, respectively). Through DEG analysis, 32 miRNAs were found to be differentially expressed among C, R and S groups, representing differences between both infected

and non-infected animals and heavy and light bacterial burdens resulting from a single-dose infection with SE. For these miRNAs, gga-miR-155 had the most abundant expression and it was significantly up-regulated in susceptible chickens (both S vs C and S vs R). Similarly, gga-miR-92-5p was highly up-regulated in resistant birds (R vs C and R vs S). Another highly expressed miRNA, gga-miR-1306-5p, was increased in both R and S compared with C, but with no significant difference between R and S. This suggests that these miRNAs in spleen might be involved as components of the immune response to SE. These results of the present study also suggested that deep sequencing technology has utility in the discovery of functional miRNAs, including those expressed at low levels, in the SE pathogenic processes. Also in this study, 3 groups were defined to increase the power of detecting miRNA DE, according to the severity of clinical symptoms and host carrier-state level (quantified as cfu / unit volume of blood), allowing comparisons to be made between birds demonstrating resistance or vulnerability to SE, in addition to simply comparing challenged and non-challenged birds. This is clearly a useful approach to identify the candidate genes involved to host resistance to SE. The present study of splenic miRNA and mRNA profiles from chickens after *Salmonella* challenge has identified differential expression of several miRNAs linked to immune responses, including miR-155, miR-9, miR-30 which have been reported previously and several miRNAs, such as miR-101-3p and miR-130b-3p, which were shown here to be associated with the immune response to infection with SE.

It is useful to predict miRNA function and construct regulation networks by the prediction of their targets and annotation of their biological function. Two immune-related KEGG pathways and one biological process were significantly enriched: Apoptosis, NOD-like receptor signaling pathway, and adaptive immune response. Interestingly, apoptosis pathway and regulation of inflammatory response were mainly enriched in the S vs C comparison, while NOD-like receptor pathway and defense response to bacterium were enriched in the R vs C comparison. These results indicated that miRNAs may play different regulatory roles associated with the extent of pathogen load in response to infection with SE, that is, between the susceptible and resistant birds.

Through the integration of miRNA and mRNA expression data and miRNA-RNA target prediction analysis, a number of putative miRNA-mRNA interactions were identified. Since hub nodes have been found to play important roles in many networks (He and Zhang, 2006), the presence of hub miRNAs was sought and, several were identified including gga-miR-155 and gga-miR-101-3p (Fig. 4-3). It has been shown that miR-155 is involved in the TLRs signaling pathway and play important roles in the innate immune response (Elton et al., 2013; Li and Shi, 2013; Quinn and O'Neill, 2011). In contrast, gga-miR-101-3p has not been previously linked to *Salmonella* infection; the present finding in chicken spleen is novel.

The leucine-rich repeat (LRR) containing protein (LRRC) 59/p34 is a type II transmembrane protein with a short C-terminal domain facing the lumen of the endoplasmic reticulum (ER) and four LRRs and coiled-coil domain facing the cytosol. LRRC59 resides in the ER and nuclear membrane, and is reported to have the function of nuclear import of fibroblast growth factor (Skjerpen et al., 2002) and CIP2A (Pallai et al., 2015) at the nuclear membrane. Although little is known about the function of LRRC59, it is becoming clear that this family of protein, could have far-reaching effects on the immune response. A recent study showed that LRRC59 dependent trafficking of nucleic acid-sensing TLRs might be beneficial for augmentation of antimicrobial immune responses from the endoplasmic reticulum via association with Uncoordinated 93 homolog B1 (UNC93B1) (Tatematsu et al., 2015). MiR-155 has been reported to play important roles in both innate and adaptive immunity in mammals. Its expression is up-regulated after activation of the innate response in murine macrophages by lipopolysaccharide, CpG and poly (I:C) and it can down-regulate these signaling pathways by targeting key signaling molecules (Elton et al., 2013; Y. Li and Shi, 2013; Maudet et al., 2014; Olivieri et al., 2013). In the current study, gga-miR-155 was significantly induced by SE infection, which was consistent with the above mammalian studies. Interestingly, the expression of gga-mir-155 was significantly higher in the S chickens compared with R birds. The expression of LRRC59 here was significantly down-regulated ($P = 0.02$) in S vs R chickens. The in vitro experiment showed that gga-miR-155 directly repressed the expression of *LRRC59*. These results indicate that gga-miR-155 and a targeted gene *LRRC59* are associated with determining resistance or susceptibility to SE infection.

Interferon regulatory factor 4 (IRF4) is a transcription factor of the IRF family that plays pivotal roles in the negative regulation of TLR signaling. Several previous studies have demonstrated that, in macrophages, *IRF4* negatively regulates the production of pro-inflammatory cytokines in response to TLR ligands (Honma et al., 2005; Negishi et al., 2005). IRF4 interacts with MyD88 and acts as a negative regulator of TLR signaling by competing with IRF5 (Negishi et al., 2005). It is well recognized that the innate immune response is critical to controlling the replication of pathogenic microorganisms, especially in young mammals and birds (Kawai and Akira, 2011; Keestra et al., 2013). In this study, the expression of *IRF4* was significantly up-regulated in S compared to uninfected C birds (FC = 1.92, $P = 0.03$) and in S vs R comparisons (FC = 2.62, $P < 0.01$). The expression of gga-miR-101-3p was significantly down-regulated in S vs C ($P < 0.01$). In addition, gga-miR-101-3p directly inhibited *IRF4* expression in vitro.

Based on the foregoing observations and interpretations, it is reasonable to propose that gga-miR-155 and gga-miR-101-3p contribute to SE-induced pathogenesis and is involved in TLR signaling pathways through directly down-regulating *LRRC59* and up-regulating *IRF4* genes, respectively.

5. Conclusion

A total of 32 DE miRNAs were identified among 3 phenotypic groups of chickens consisting of non-challenged controls, birds that were resistant to challenge with SE, and those that were susceptible to SE with heavy pathogen loads at 24 h after infection. Two miRNAs, gga-miR-155 and gga-miR-101-3p, could directly alter the expression of target IRF4 and LRRC59, respectively. These investigations indicate that miRNAs in spleen play a major role in the SE infection process. The findings will facilitate understanding resistance and susceptibility to *Salmonella* infection through miRNA-induced systems, and may assist breeding for genetic resistance to SE in poultry.

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5

Determination of the optimum Concentration of dietary FOS against *Salmonella* Infection

Controlling and reducing Salmonella colonization as well as the expression of pro-inflammatory cytokinesis are important indices evaluating the effect of FOS against Salmonella infection. In this chapter, the optimum concentration of dietary FOS was based on these indicators in chicks under Salmonella infection.

From Peng Li, Weiwei Fan, Nadia Everaert, Ranran Liu, Qinghe Li, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. 2018. Effect of dietary fructooligosaccharide on reducing the inflammatory response in young chickens infected with *Salmonella* Enteritidis (*submitted).*

Abstract The effects of supplemental fructooligosaccharides (FOS) on inflammatory responses in spleen and cecal tonsils of chicks challenged with *Salmonella* Enterica (SE) were investigated. One day-old specific-pathogen-free (SPF) White Leghorn chicks were randomly allocated to four treatment groups, each with two cages of 40 birds and fed a basal diet with 0, 10, 20, and 30 g/kg FOS throughout. Three days post-hatch, all chicks were orally challenged with 10^8 colony-forming unit SE and serum, liver, spleen and cecal tissues were collected at 0.5, 1, 2, 7, and 14 days post-infection (dpi). Diets containing 1% and 2%, but not 3%, FOS reduced numbers of SE in cecum and liver compared to the basal diet ($P < 0.01$) at 7 and 14 dpi. FOS supplementation (1% and 2%) decreased ($P < 0.01$) serum levels of interleukin-1β at all sampled times. Similarly, dietary FOS (1% and 2%, but not 3%), decreased the relative splenic expression of lipopolysaccharide-induced tumor necrosis factor (TNF) factor (*LITAF*) and interleukin-6 (*IL-6*) at 2, 7 and 14 dpi and cecal expression of *LITAF* and *IL-6* was reduced at 7 dpi ($P < 0.01$). In conclusion, dietary supplementation with 1 or 2% FOS alleviated pathogen colonization and SE-induced inflammation, evidenced in serum and peripheral immune organs of young chickens.

Key words: Fructooligosaccharides, *Salmonella* Enteritidis, pro-inflammatory cytokines, spleen and cecal tonsils.

1. Introduction

Inflammation is a defense response against infection, designed to clear foreign pathogens and to inhibit their detrimental effects. In response to systemic infection of chickens with *Salmonella*, pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and lipopolysaccharide-induced tumor necrosis factor (TNF) factor (LITAF), all host-produced by the innate and adaptive immune system (Chappell et al., 2009; Setta et al., 2012; Tohidi et al., 2018), are critical for the control of bacterial growth. Unfortunately, during the early stages of acute *Salmonella* infection or stimulation with high doses of LPS, excessive production of pro-inflammatory cytokines can lead to endotoxic shock or sepsis-related deaths (Cohen, 2002; Kurtz et al., 2017; Netea et al., 2017), especially in young animals. Thus, reducing the complications caused by acute inflammatory stimuli while maintaining a moderate degree of inflammation is beneficial to the host resistance to infection.

Fructooligosaccharides (FOS) are short-chain non-digestible carbohydrates extracted from plant sources (e.g., chicory root, onion, beet and cane sugar) and are considered to have a prebiotic effect in maintaining intestinal health (Meyer and Stasse-Wolthuis, 2009; Sarao and Arora, 2017), improving growth performance, and modulating immune function in animals (Flickinger et al.,, 2003; Shang, 2014; Tran et al., 2016). Although FOS-inulin addition has been shown to depress the expression of pro-inflammatory cytokines in vitro and in vivo (Babu et al., 2012; Ferenczi et al., 2016; Ortega-González et al., 2014), little information is available describing the roles of dietary FOS on inflammation, especially in peripheral immune organs of chicks. Similarly, the effect of a FOS-supplemented diet in potentially alleviating induced inflammation in *Salmonella*-exposed chicks is warranted. This study was therefore conducted to determine if dietary FOS alleviated the inflammation in spleen and cecal tonsils of chickens challenged with *Salmonella* Enteritidis.

2. Materials and Methods

2.1 Experimental design

The experimental animal protocol was approved by the Animal Care and Use Committee of China Agricultural University, Beijing, PRC. A total of 200 specific-pathogen-free (SPF) White Leghorn chicks were supplied by the Beijing Laboratory Animal Research Center (BLARC). One day-old chicks were randomly allocated into 4 treatment groups, each with 2 replicates, and reared in 8 separate cages (25 birds / cage) in the SPF animal experiment center of China Agricultural University. The 4 treatments were 0, 10, 20, and 30 g/kg of FOS (F8052, purity ≥ 90%; Sigma-Aldrich, St. Louis, MO) added to a corn-soybean-based diet (Beijing Keaoxieli Feed Co., Ltd, BLARC). Details of the basal and FOS-supplemented diets, all satisfying recommended critical levels of nutrients (NRC, 1994), are given in Table 5-1.

Animals were provided ad libitum access to water and the 4 diets throughout. The feed was sterilized by Co^{60} irradiation and the water was previously sterilized at 121℃ for 15 min. The temperature in the isolator was kept at 35℃ in the first week and declined 2℃ each week until the end of the experiment. Three days post-hatch, all chickens were orally challenged with 1mL phosphate-buffered saline (PBS) containing 10^8 cfu *Salmonella enterica* serovar Enteritidis (CMCC50041, China Institute of Veterinary Drugs Control, Beijing).

Five randomly selected chickens from each cage were euthanized at 0.5, 1, 2, 7, and 14 days post-infection (dpi). Blood was collected, allowed to clot and serum was stored at -20℃. The spleen and cecal tonsils were removed and portions were snap-frozen in liquid nitrogen for RNA extraction. The liver and cecum were removed aseptically for bacteriological examination.

2.2 Bacteriological examination

The liver and cecum tissues were aseptically removed at each time post-infection (5 birds per group). Then liver (500 mg) and cecum (2.0-cm section, no content) were homogenized using Tissuelyser (JX Co., Ltd, Shanghai) in 5 mL PBS. Quantification of SE burden within tissue samples was performed as described by Kaiser and Lamont (2001, 2002). These homogenates (200 μL) above were enriched in selenite broth (Oxoid, Basingstoke, UK), for 24 h at 37°C. Each enrichment culture was screened for presence of SE by plating on brilliant green agar (BGA) (Fisher Scientific, Pittsburgh, PA) plates containing 100 μg / mL nalidixic acid (Oxoid, Basingstoke, UK) and then incubating for 24 h at 37℃. If colony morphological identification of SE was questionable, the colony identity was confirmed by *Salmonella* antiserum group D agglutination (Fisher Scientific, Pittsburgh, PA). Enrichment cultures from samples that produced SE-positive plates were serially 10-fold diluted and plated in triple on BGA plates to quantify *S.* Enteritidis colony-forming units (cfu) per milliliter.

2.3 Measurement of serum IL-1β

Concentrations in serum of interleukin 1β (IL-1β) were determined using commercial chicken competitive ELISA kits (CUSABIO, Wuhan, PRC).

2.4 Total RNA extraction and reverse transcription

Spleen and cecal tonsil was used for relative abundance of inflammatory genes. Total RNA was extracted from the tissues using MiniBEST Universal RNA Extraction Kits (TaKaRa, Dalian, PRC), quantified by OD260 (NanoDrop spectrophotometer ND-1000, Thermo Fisher Scientific, Waltham, MA) and purity was assessed by OD260/280. Approximately 100 μg of RNA was used for reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

2.5 Quantitative real-time PCR (qPCR)

Table 5-1. Composition and nutrient levels of basal and supplemented diets (air-dry basis) [g/kg].

	Diets			
Components and analyses	0 FOS*	1% FOS	2% FOS	3% FOS
Ingredients				
Corn	680.1	660.2	640.7	621.3
Choline chloride	0.3	0.3	0.3	0.3
Soybean meal	275.6	279.6	283.5	287.2
Corn oil	0	5.8	11.4	17
Salt	2	2	2	2
Limestone powder	4.8	4.8	4.8	4.8
Calcium dihydrogen phosphate	16	16	16	16
Cystine	3	3.1	3.1	3.1
Methionine	0.2	0.2	0.2	0.2
Vitamin premix†	10	10	10	10
Microelement premix‡	5	5	5	5
Feed grade silicondioxide/titanium	3	3	3	3
FOS	0	10	20	30
Calculated nutrient level				
ME, [MJ/kg]	11.98	11.98	11.98	11.98
CP	181.0	181.0	181.0	181.0
Available phosphorus	4.0	4.0	4.0	4.0
Calcium	9.0	9.0	9.0	9.0
Lysine	9.1	9.1	9.1	9.0
Methionine	3.1	3.1	3.0	3.1
Cystine	6.2	6.2	6.2	6.2

*Control group without FOS supplementation, FOS = fructooligosaccharides;
†Vitamin premix provided the following per kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 3000 IU; vitamin E, 25 IU; nicotinic acid, 60 mg; vitamin B12, 18 µg; calcium

pantothenate, 25 mg; vitamin K3, 4 mg; thiamin, 3.0 mg; riboflavin, 8.0 mg; vitamin B6, 7.0 mg; folic acid, 2 mg; biotin, 0.2 mg.

‡Microelement premix provided the following per kilogram of diet: Fe, 100 mg; Cu, 8 mg; Mn, 120 mg; Zn, 100mg; I, 0.7 mg; Se, 0.3 mg.

Transcripts of *IL-6* and *LITAF* in the spleen and cecal tonsils were quantified by qPCR in an ABI 7500 Detection System (Applied Biosystems, Foster, CA). Amplification was performed in triplicate in a total volume of 20 µl, containing 10 µl of 2 × KAPA SYBR FAST qPCR Master Mix (Roche, Basel, Switzerland), 1 µl of the diluted cDNA, 0.5 µl of each primer, and 0.4 µl ROX Low and 7.6 µl PCR-grade water. The qPCR program started with denaturing at 95℃ for 3 min, followed by 40 cycles of 95℃ for 3 s and 60℃ for 34 s. Data were analyzed with ABI 7500 SDS software (ABI) with the baseline being set automatically by the software. Relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The qPCR primers used here were: *IL-6* (sense 5'-TTCGACGAGGAGAAATGCCT / antisense 5'-CGACGTTCTGCTTTTCGCT-AT), *LITAF* (sense 5'-TGTGTATGTGCAGCACCCGTAGT / antisense 5'-GGCAT-TGCAATTTGGACAGAAGT), *β-actin* (sense 5'-GAGAAATTGTGCGTGACATC A / antisense 5'-CCTGAACCTCTCATTGCCA). Annealing temperature is 60℃. *β-actin* was used as the housekeeping gene, as in previous chicken studies with *Salmonella* challenge (Li et al., 2017; Li et al., 2010).

2.6 Statistical analysis

The results are presented as means ± SEM. The main effects of diet, time post-SE challenge, and their interaction were assessed by 2-way ANOVA using the GLM procedure of SAS 9.2 (SAS Inst. 2008). When interactions were apparent ($P < 0.05$ for this determination), treatment means were compared, within sampling times, using Duncan's multiple range test and $P < 0.05$.

3.Results

3.1 Effects of dietary FOS on S. Enteritidis colonization in chicken cecum and liver

As shown in Table 5-2, at 7 and 14 dpi, there was a significant reduction ($P < 0.01$) in SE counts recovered from cecum and liver of chicks supplemented with 1% and 2% FOS compared to those fed no FOS. The diet with 3% FOS, however, had no significant effect ($P > 0.05$) on reducing the SE burden in the cecum at the same times, but was beneficial at 2 and 14 dpi in liver.

3.2 Effects of dietary FOS on serum IL-1 β following S. Enteritidis infection

As shown in Figure 5-1, FOS supplementation (1% and 2%) significantly decreased ($P < 0.05$) serum concentrations of IL-1β at all times post-infection, as compared with chicks not given FOS; this was also true of 3% FOS, except at 14 dpi.

Table 5-2. The effect of dietary FOS on the numbers [log_{10} cfu] of *S.* Enteritidis in the cecum and liver of SPF chicks infected at 3 d of age[†].

		Days post-infection				
		0.5	1	2	7	14
Cecum	No FOS	7.86±0.12	8.20±0.10	7.74±0.10^{ab}	5.43±0.10^{a}	3.14±0.06^{a}
	1% FOS	7.64±0.15	7.82±0.15	7.29±0.13^{b}	4.11±0.07^{b}	2.28±0.10^{b}
	2% FOS	7.73±0.21	7.79±0.19	7.55±0.15^{ab}	4.14±0.08^{b}	2.01±0.06^{c}
	3% FOS	7.85±0.24	7.98±0.18	7.95±0.20^{a}	5.65±0.09^{a}	3.13±0.08^{a}
Liver	No FOS	4.39±0.11	4.39±0.10	4.96±0.11^{a}	4.01±0.11^{a}	2.00±0.08^{a}
	1% FOS	4.10±0.08	4.08±0.10	4.11±0.06^{c}	3.08±0.06^{b}	0.75±0.09^{c}
	2% FOS	4.06±0.08	4.05±0.10	4.12±0.09^{c}	3.08±0.08^{b}	0.88±0.06^{c}
	3% FOS	4.24±0.12	4.33±0.16	4.53±0.16^{b}	3.77±0.08^{a}	1.64±0.08^{b}

[a-c]Values with different superscripts in the same column, within tissues, are significantly different ($P < 0.05$) by Duncan's multiple range test (n = 12).
[†]Chicks were inoculated with 10^8 cfu *S.* Enteritidis at 3 d of age. Diet effect: $P < 0.0001$, Time post-SE: $P < 0.0001$, diet × Time post-SE: $P < 0.0001$ (by two-way ANOVA). FOS = fructooligosaccharides; cfu = colony forming units.

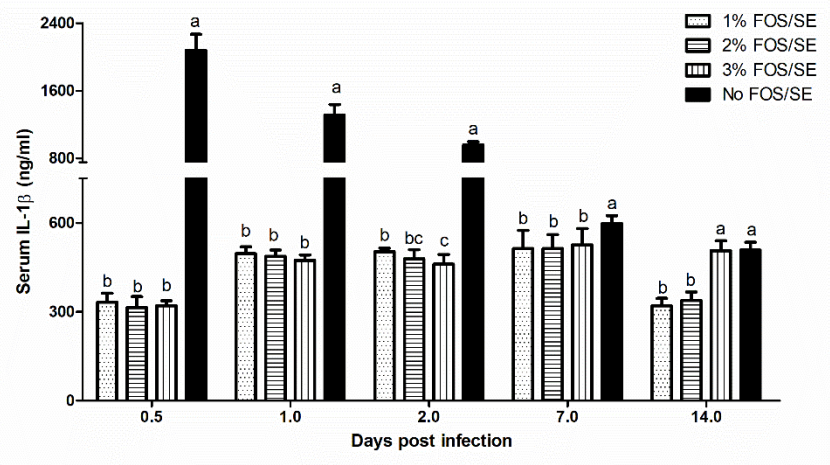

Figure 5-1. IL-1β concentrations in serum after *S.* Enteritidis challenge in chicks fed a control diet or diets supplemented with 3 levels of FOS. The data are means ± SEM (n = 12). Diet × Days post-SE interactions were significant ($P < 0.0001$, two-way ANOVA) so comparisons were confined to FOS addition, on a within-day basis. Different letters (a to c) at each day indicate significant ($P < 0.05$) differences. FOS = fructooligosaccharides; IL-1β = interleukin 1β.

3.3 Effects of dietary FOS on the expression of proinflammatory cytokines in spleen and cecal tonsils following S. Enteritidis infection

As compared with chicks fed the diet without FOS, those receiving 1% and 2% FOS supplementation had a decreased ($P < 0.01$) abundance of *IL-6* and *LITAF* transcripts in the spleen at 2, 7 and 14 dpi (Figure 5-2A, 3A), except for *LITAF* gene expression in 2% FOS-treated birds at 14 dpi. Although the relative expression of *LITAF* was decreased ($P < 0.01$) at 2, 7 and 14 dpi in cecal tonsils of FOS-treated birds (Figure 5-3B), the expression of *IL-6* was significantly reduced only at 7 dpi (Figure 2B). In addition, there was a higher expression of *IL-6* in cecal tonsil at 2 dpi in chicks given FOS compared to non-treated birds.

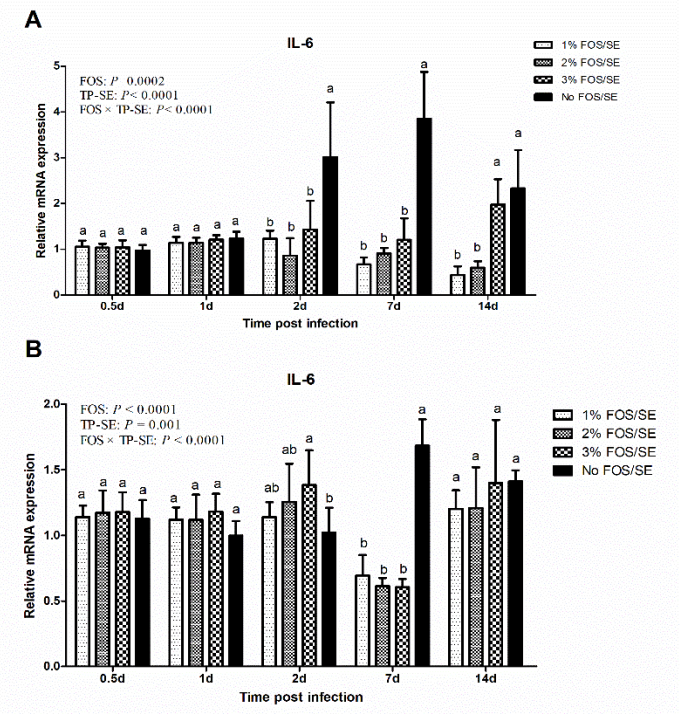

Figure 5-2. Relative abundance of *IL-6* transcripts in spleen (A) and cecal tonsils (B) after *S.* Enteritidis challenge in chicks fed a control diet or diets supplemented with 3 levels of FOS. The data, all normalized to β-actin, are means ± SEM (n = 12). Significant differences ($P < 0.05$), on a within-dpi basis, are indicated by different letters (a to c). FOS = fructooligosaccharides; IL-6 = interleukin 6.

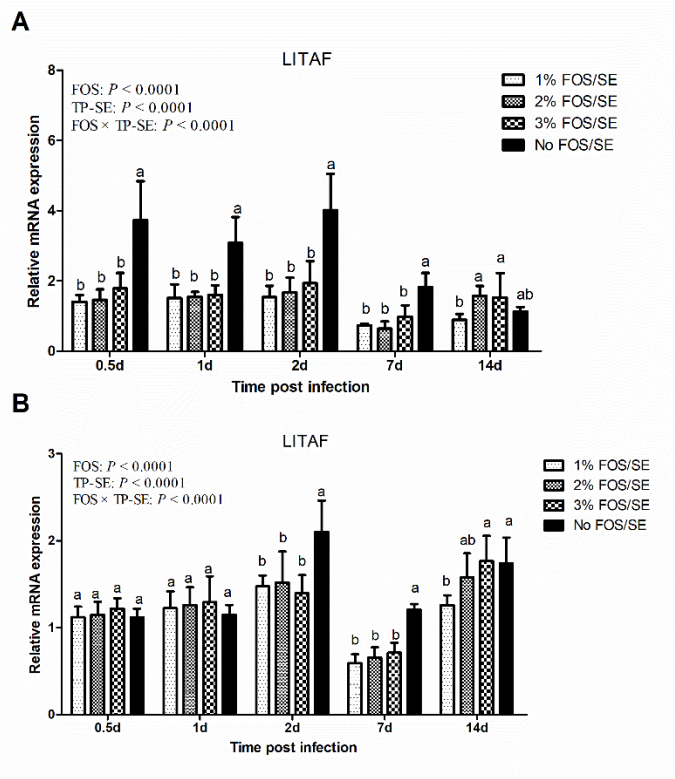

Figure 5-3. Relative abundance of *LITAF* transcripts in spleen (A) and cecal tonsils (B) after S. Enteritidis challenge in chicks fed a control diet or diets supplemented with 3 levels of FOS. The data, all normalized to β-actin, are means ± SEM (n = 12). Significant differences ($P < 0.05$), on a within-dpi basis, are indicated by different letters (a to c). FOS = fructooligosaccharides; LITAF = lipopolysaccharide-induced tumour necrosis factor (TNF) factor.

4. Discussion

Complex interactions exist among dietary factors and immune functions in conferring protection against pathogens and other health challenges. For infectious diseases, it is useful to appreciate that many infectious diseases are not caused directly by the pathogen, but by an over-exuberant innate immune response to its presence (Eckmann and Kagnoff, 2001). Interestingly, diets supplemented with prebiotics have direct effects on disease vectors and indirect immunomodulatory effects by depressing host production of pro-inflammatory cytokines (Ferenczi et al., 2016; Vogt et al., 2015; Weitkunat et al., 2015). Little present known of the roles of dietary FOS regulating inflammation in chicks. As first reported here, diets supplemented with low amounts of FOS decreased SE colonization in chicks and

reduced production of pro-inflammatory cytokines, as evidenced by serum concentrations and gene expression in peripheral immune organs.

Supplementation of diets with FOS has positive modulatory effects on the immune system in chickens, such as increasing the titers of plasma IgM and IgY (Janardhana et al., 2009), enhancing general resistance (sheep red blood cells and heterophil / lymphocyte ratio) (Emami et al., 2012; Kim et al., 2011), and regulating the level of pro-inflammatory cytokines (Schley and Field, 2002; Shang et al., 2015). Recent studies showed that FOS-inulin alleviated mucosal damage through decreasing gene expression of *TNF-α*, *IL-1α*, *IL-1β*, and *iNOS* in rat mucosal tissue (Ma et al., 2018; Wilson and Whelan, 2017), and increased phagocytic activity and secretory capacity against LPS or SE challenge of immune cells (Kupffer cells and macrophages) (Babu et al., 2012; Capitán-Cañadas et al., 2014; Neyrinck et al., 2004). In addition, FOS-treated mice also showed lower mortality and incidences of aberrant crypt foci than did control mice when exposed to dimethylhydrazine or *Salmonella* Typhimurium (Buddington et al., 2002). Co-culture experiments of *Salmonella* Enteritidis with chicken HD11 macrophages showed that FOS significantly decreased IL-1β expression, suggesting that FOS-inulin can modulate the innate immune system by reducing activation of inflammasomes (Babu et al., 2012). Ortega-González et al. (2014) reported that exposure to FOS-inulin, to a lesser extent, reduced the secretion of the inflammatory cytokines IL-6, IL-10 and TNF-α from rat intestinal cells (IEC18) and macrophages.

In the present study, dietary supplementation with FOS (1% and 2%) decreased serum levels of IL-1β at all times post-infection with SE. Dietary FOS (1% and 2%, but not 3%), decreased gene expression of pro-inflammatory cytokines *LITAF* and *IL-6* in the spleen at 2, 7 and 14 dpi. Although the relative expression of *LITAF* decreased at 2, 7 and 14 dpi in cecal tonsils of chicks given FOS, expression of *IL-6* was reduced only at 7 dpi. In contrast, a study using rat monocytes (Capitán-Cañadas et al., 2014) showed that FOS-inulin induced the release of TNF-α, growth-regulated oncogene α, and IL-10. Shang et al. (2015) found that supplementation with FOS also increased the ileal expression of *IL-1β*, *IL-10* and interferon (IFN)-γ) of birds under SE infection. These inconsistent results may be related to experimental treatment, purity degree and does of FOS/inulin addition and rearing condition, as well as target tissues.

Controlling and reducing *Salmonella* colonization is an important measure to reduce tissue damage and allergic reactions in the host, especially in young animals. Results from pathogen challenged broilers given FOS suggested a reduced susceptibility to either *Salmonella* spp. or Escherichia coli infection (Bailey et al., 1991; Oyarzabal and Conner, 1996; Telg and Caldwell, 2009). Here, diets with 1% and 2% but not 3% FOS reduced the number of bacteria in the liver and cecum after SE challenge at 4, 7 and 14 dpi. These results indicate that the higher level of FOS

was not conducive for the host being able to control the bacterial proliferation. FOS, added to chicken diets, may reduce *Salmonella* colonization, an antibiotic effect, likely resulting from fermentation of FOS by *Bifidobacteria* (Flickinger et al., 2003).

5. Conclusion

The present study demonstrates the beneficial effect of adding FOS, probably at 1%, to the diet of young chicks. It clearly reduced SE colonization from 4 to 14 dpi with *Salmonella* Enteritidis and reduced the extent of the pro-inflammatory response, as reflected by circulating IL-1β and relevant gene expression in spleen (*IL-6*) and cecal tonsil (*IL-6* and *LITAF*).

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6

FOS modulating the host immune Response to *Salmonella* infection

The aim of this Chapter was to investigate whether FOS addition alters the expression of inflammatory genes involved in TLR4-MyD88-dependent signaling following Salmonella infection.

From Peng Li, Weiwei Fan, Nadia Everaert, Ranran Liu, Qinghe Li, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. 2018. Effect of fructooligosaccharide on modulating the expression of inflammatory genes involved in MyD88-dependent signaling following *Salmonella* infection in young chickens (*submitted).*

Abstract: The effects of fructooligosaccharides (FOS) on inflammatory responses in chicks and chicken macrophage challenged with *Salmonella* Enteritidis (SE) / lipopolysaccharide (LPS) were investigated. In vivo, one day-old specific-pathogen-free (SPF) White Leghorn chicks were randomly allocated to 4 treatments, each with 2 cages of 12 birds and were fed a basal diet without or with 10 g/kg FOS throughout. Three days post-hatch, birds from each subgroup of the two treatment groups were orally challenged with 10^8 colony-forming unit (cfu) SE or saline. Spleen and cecal tonsils were collected at 1, 7 and 14 days post-infection (dpi). Meanwhile the direct interactions of FOS/LPS on chicken macrophages were also examined in vitro. Results showed that dietary FOS significantly reduce the expression of proinflammatory cytokine *IL-6* ($P < 0.05$) and *TNF-α* ($P < 0.05$), as well as the abundance of inflammation-related pathway genes *TLR4* ($P < 0.05$), *MyD88* ($P < 0.05$), *TRAF6* ($P < 0.05$) and *NF-κB* ($P < 0.05$) in spleen at 7 and 14 dpi, and in cecal tonsils at 14 dpi during *S.* Enteritidis infection in young chickens. Moreover, suitable concentration (200μg/mL) of FOS treatment can increase direct the expression of *IL-6* ($P < 0.05$) and *TNF-α* ($P < 0.05$) *in vitro* on chicken macrophage and alleviated the abundance of *TNF-α* ($P < 0.05$) and *IL-6* ($P < 0.05$) under LPS exposed. In addition, the immunomodulatory effect of FOS on the expression of inflammatory cytokines and signaling genes exhibited the dose and time-dependent manner. Taken together, our findings provided novel information about the prebiotics may regulate the MyD88-dependent signaling in avian species, which maybe the possible mechanism of early intervention in anti-*Salmonella* infection.

Key words: Fructooligosaccharides, *Salmonella* Enteritidis, proinflammatory cytokines gene expression, MyD88-dependent signaling; spleen and cecal tonsils, chicken macrophage.

1. Introduction

Salmonella Enteritidis (SE) and Typhimurium (ST) are facultative intracellular pathogens that can colonize chickens, contaminating meat and eggs and thereby constituting an insidious risk for public health (Barrow et al., 2012; Calenge and Beaumont, 2012; Kogut and Arsenault, 2017). SE is among the top ranking food-borne pathogens causing huge economic and human life losses. For food animals, therapeutic antibiotics are used to control economically important infections. Recent concerns of bacterial antibiotic resistance and the presence of antibiotic residues in meat have led to alternative methods such as dietary interventions being evaluated as a means of reducing or eliminating *Salmonella* colonization in chickens (Babu and Raybourne, 2008). Notable among the interventions are the use of prebiotics and direct-fed microbials (DFM) in animal feeds as they have been shown to have immunomodulatory effects by boosting the host immune response and thereby conferring resistance to infections.

Fructooligosaccharides (FOS) are short-chain non-digestible carbohydrates extracted from plant sources (e.g., chicory root, onion, beet and cane sugar) and are considered to have a prebiotic effect in maintaining intestinal health (Meyer and Stasse-Wolthuis, 2009; Sarao and Arora, 2017), improving growth performance, and modulating immune function in animals (Flickinger et al., 2003; Shang, 2015; Tran et al. 2018). It is well-known that immunomodulation mechanisms of FOS-inulin encompass stimulation of growth of lactic acid bacteria, but can also be mediated by fermentation products of these bacteria i.e. short chain fatty acids (SCFA). Recent in vitro studies have demonstrated that FOS-inulin can be recognized directly by intestinal epithelial cell lines (Zenhom et al., 2011; de Kivit et al., 2011) and immune cells (Babu et al. 2012; Ortega-González et al., 2014; Capitán-Cañadas et al. 2014), with inhibition of the NF-κB signaling pathway and eventually reducing production of pro-inflammatory cytokines. In addition, in TLR4-KO mice, FOS/inulin-induced secretion of monocyte chemoattractant protein 1 (MCP-1) was also reduced in colonic explants compared with the untreated controls (Capitán-Cañadas et al. 2014).

During *Salmonella* invasion of tissue, lipopolysaccharide (LPS) in its cell wall is protectively recognized by TLRs (TLR4 and TLR5), triggering a NF-κB-mediated inflammatory response and producing pro-inflammatory cytokines such as interleukin-6 (IL-6), leading to an influx of heterophils and macrophages to the site of infection, especially in the immune tissues (Van Immerseel et al., 2002; Munyaka et al., 2013; Wigley, 2014). Compared with mammals, the chicken MyD88-dependent pathway has unique roles in host immune response to SE infection because of the absence of any MyD88-independent pathway (Keestra et al., 2008, 2013). Although the potential effect of FOS-inulin on regulating the immune response has been widely addressed in humans and rats (Ferenczi et al. 2016; Ortega-

González et al. 2014), little information is available in chicks. Thus, the aim of the present study was to investigate whether FOS supplementation alters the expression of inflammatory genes involved in MyD88-dependent signaling following *Salmonella* infection in young chickens. Direct actions of FOS/LPS on chicken macrophages were also examined.

2. Materials and Methods

2.1 Bacterial strains, FOS and Diets

Salmonella enterica serovar Enteritidis, CMCC50041, was obtained from the China Institute of Veterinary Drugs Control (IVDC). Bacteria were resuscitated for 18 h in Luria-Bertani (LB) broth at 37℃ in an orbital shaking incubator at 150 rpm. The number of colony-forming units (cfu) of *S.* Enteritidis was determined by plating serial dilutions. FOS from chicory was obtained from Sigma (F8052, purity ≥ 90%; Sigma-Aldrich, St. Louis, MO). The length of the fructose chain varies from 2 to 60 with an average degree of polymerization of > 10. All feed was provided by Beijing Keaoxieli Feed Co., Ltd (BLARC). The feed was sterilized by Co^{60} irradiation and the water was previously sterilized at 121℃ for 15 min.

2.2 Animal experimental design

The experimental animal protocol was approved by the Animal Care and Use Committee of China Agricultural University, Beijing, PRC. The prebiotics were administered to chicks by feeding, starting on the day of hatch. Because 1% FOS-supplementation was shown previously (P Li, W Sun, N Everaert and J Wen, unpublished results) to be efficacious on the response to SE in chicks, the same experimental diet was used here to assess the effect on the immune response against *Salmonella* challenge. A total of 96 specific-pathogen-free (SPF) White Leghorn chicks were supplied by the Beijing Laboratory Animal Research Center (BLARC). One-d-old chicks were randomly allocated to 4 treatments, each with 2 cages of 12 birds in the SPF animal experiment center of China Agricultural University. The 4 treatments were: (1) chicks fed the basal diet; (2) chicks fed the basal diet supplemented with FOS; (3) chicks fed the basal diet then challenged with SE; and (4) chicks fed the basal diet supplemented with FOS then challenged with SE. Details of the basal and FOS-supplemented diets, all satisfying recommended critical levels of nutrients (NRC, 1994), are given in Table 6-1. Animals were given ad libitum access to water and the 4 diets throughout. The temperature in the isolator was kept at 35℃ in the first week and declined by 2℃ each week until the end of the experiment. On d 3 post-hatch, birds were orally administered 1mL vehicle PBS alone (Groups 1 and 2), or PBS containing 10^8 cfu *S.* Enteritidis (Groups 3 and 4). Six randomly selected chickens from each cage were slaughtered at 1, 7 and 14 d

post-infection (dpi). The spleen and cecal tonsils were removed and portions were snap-frozen in liquid nitrogen for RNA extraction.

Table 6-1. Composition and nutrient levels of basal and supplemented diets (air-dry basis) [g/kg].

	Diets	
Components and analyses	0 FOS*	1% FOS
Ingredients		
Corn	680.1	660.2
Choline chloride	0.3	0.3
Soybean meal	275.6	279.6
Corn oil	0	5.8
Salt	2	2
Limestone powder	4.8	4.8
Calcium dihydrogen phosphate	16	16
Cystine	3	3.1
Methionine	0.2	0.2
Vitamin premix†	10	10
Microelement premix‡	5	5
Feed grade silicondioxide/titanium	3	3
FOS	0	10
Calculated nutrient level		
ME, [MJ/kg]	11.98	11.98
CP	181.0	181.0
Available phosphorus	4.0	4.0
Calcium	9.0	9.0
Lysine	9.1	9.1
Methionine	3.1	3.1

Cystine	6.2	6.2

*Control group without FOS supplementation, FOS = fructooligosaccharides;
†Vitamin premix provided the following per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 3000 IU; vitamin E, 25 IU; nicotinic acid, 60 mg; vitamin B_{12}, 18 μg; calcium pantothenate, 25 mg; vitamin K_3, 4 mg; thiamin, 3.0 mg; riboflavin, 8.0 mg; vitamin B_6, 7.0 mg; folic acid, 2 mg; biotin, 0.2 mg.
‡Microelement premix provided the following per kilogram of diet: Fe, 100 mg; Cu, 8 mg; Mn, 120 mg; Zn, 100mg; I, 0.7 mg; Se, 0.3 mg.

2.3 In vitro experimental design

Chicken macrophage-like cells, HD11, were grown at 37°C with 5% CO2 in RPMI-1640 medium that contained 10 mM HEPES, 1 mM sodium pyruvate, 1% glutamine, 1% MEM NEAA, 10% fetal bovine serum and 5% chicken serum (all reagents from Gibco, Grand Island, NY). To examine the expression of inflammatory genes (*TNF-α* and *IL-6*), HD11 cells were treated with FOS (10, 50, 100, and 200 μg / mL) for 12 h in 24-well plates. Cells were collected and snap frozen at -80°C. The highest safe dose (200 μg / mL) of FOS addition was determined by Babu et al. (2012).

For the LPS challenge experiments, cells were plated at a density of 6×10^6 cells/well in 24-well plates and incubated with FOS (0, 10, 50, 100, and 200 μg/mL) for 6h, then were stimulated with LPS (*Escherichia coli* 055:B5, Sigma) at a final concentration of 10 μg/mL; treatments were imposed on triplicate wells. Cells were harvested for RNA extraction at 0, 3, 6, 12, and 24 h post-treatment and relative abundance of inflammatory genes was measured by real-time RT-PCR.

2.4 RNA isolation and measurement of gene expression by real-time RT-PCR

Total RNA was extracted from the tissues or HD11 cells using MiniBEST Universal RNA Extraction Kits (TaKaRa, Dalian, PRC). RNA was quantified using the NanoDrop ND-2000 spectrophotometer (NanoDrop Products, Wilmington, DE). cDNA was synthesized from 100 μg RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA), and real-time PCR was carried out as previously described (Li et al., 2017). The primers of the target genes were as used previously (Li et al., 2010) or newly designed (Table 6-2). Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *β-actin* was used as the reference gene, as in previous studies with *Salmonella* challenge of chickens (Li et al. 2017; Li et al. 2010).

Table 6-2. Primers used in the real time PCR.

Genes	[1]Sequence (5'-3')	Accession No.
IL-6	F: TTCGACGAGGAGAAATGCCT	NM_204628
	R: CGACGTTCTGCTTTTCGCTAT	
TNF-α	F: TGTGTATGTGCAGCACCCGTAGT	XM_015294125
	R: GGCATTGCAATTTGGACAGAAGT	
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT	AY064697
	R: GCGACGTTAAGCCATGGAAG	
MyD88	F: TGATGCCTTCATCTGCTACTG	EF011109
	R: TCCCTCCGACACCTTCTTTCTA	
TRAF6	F: GACTTGGATAGTGGCTGCTG	XM_421089
	R: TCCCTGCGTCTCCTCTGTGA	
NF-κB	F: CAGCCCATCTATGACAACCG	NM_205129
	R: TCAGCCCAGAAACGAACCTC	
β-action	F: GAGAAATTGTGCGTGACATCA	L08165
	R: CCTGAACCTCTCATTGCCA	

[1]F = forward; R = reverse. Primers for *TLR4*, *MyD88*, *TRAF6*, *NF-κB* and *β-action* were used in our previous study (Li et al., 2010).

2.5 Statistical analysis

For the animal experiment, the main effects of diet, SE challenge, and their interaction were assessed at each of the 3 sampling times by 2-way ANOVA using the GLM procedure of SAS 9.2 (SAS Inst. 2008). When interactions were significant ($P < 0.05$), means were compared by Duncan's multiple range tests. Data are means with the SEM derived from the ANOVA error mean square for n = 8. For the in vitro experiments, one-way ANOVA was used to assess the effect of treatment for 12 h on the inflammatory response; each mean was compared to the control (no exposure to FOS or LPS). Subsequently, the time-course and magnitude of response to LPS was assessed after pre-treating cells with increasing concentrations of FOS. Results are expressed as fold-change in transcript number (means ± SEM, n = 3) relative to the LPS-exposed cells without any FOS pre-treatment. Significance was defined as $P < 0.05$.

3. Results

3.1 Effects of FOS on expression of inflammatory genes involved in MyD88-dependent signaling in spleen

As shown in Table 6-3, the expression of pro-inflammatory genes *IL-6* and *TNF-α* was upregulated ($P < 0.05$) in spleen following SE infection at 1, 7 and 14 d post-infection (dpi). In contrast, treatment with FOS+SE reduced the abundance of *IL-6* and *TNF-α* transcripts ($P < 0.05$), when compared with positive control SE chicks at 7 and 14 dpi. As expected, SE challenge upregulated the expression of the MyD88-dependent signaling genes *TLR4*, *MyD88*, *TRAF6* and *NF-κB* ($P < 0.05$) when compared with untreated controls at all times post infection except *Myd88* at 1 dpi, whereas treatment with FOS+SE reduced expression of those genes compared with control chicks at 14 dpi ($P < 0.05$). Strikingly, a treatment effect of dietary FOS ($P < 0.01$) was noted in the abundance of splenic transcripts of inflammation-related genes at 7 and14 dpi.

3.2 Effects of FOS on expression of inflammatory genes involved in MyD88-dependent signaling in cecal tonsils

Similar in spleen, dietary FOS increased the expression of *IL-6* and *TNF-α* ($P < 0.05$) in the cecal tonsils of non-infected birds at 7 and 14 dpi (Table 6-4). Only at 14 dpi, however, treatment with FOS+SE reduced the expression of *IL-6* and TNF-α ($P < 0.05$) compared with positive control SE chicks. In addition, expression of the MyD88-dependent signaling genes *TLR4*, *MyD88*, *TRAF6* and *NF-κB* was upregulated ($P < 0.05$) when compared with untreated control chicks at 7 and 14 dpi, and treatment with FOS+SE reduced expression of these genes at 7 and 14 dpi ($P < 0.05$), except the *TRAF6* at 7dpi. Overall, there was a treatment effect ($P < 0.01$) of dietary FOS in the transcript abundance of inflammatory and signaling genes in cecal tonsils at 14 dpi.

Table 6-3. Effects of FOS on expression of inflammatory genes involved in MyD88-dependent signaling in spleen.

Diet	SE	*IL-6*			*TNF-α*			*TLR4*			*MyD88*		
		1DPI	7DPI	14DPI	1DPI	7DPI	14DPI	1DPI	7DPI	14DPI	1DPI	7DPI	14DPI
NC	-	1.012	1.014^{c}	1.108^{b}	1.003	1.007^{c}	1.001^{c}	0.994	1.017^{c}	1.020^{c}	1.007	0.996	0.978^{c}
	+	1.238	3.349^{a}	2.327^{a}	3.075	1.824^{a}	1.554^{a}	1.254	1.855^{a}	1.302^{a}	0.876	1.59	1.280^{a}
FOS	-	1.028	1.121^{c}	1.127^{b}	1.149	1.082^{c}	1.212^{b}	1.036	1.063^{c}	1.166^{b}	0.955	0.984	1.094^{b}
	+	1.143	2.479^{b}	1.434^{b}	3.006	1.352^{b}	1.321^{b}	1.161	1.570^{b}	1.233^{b}	0.929	1.684	1.173^{b}
SEM		0.029	0.207	0.133	0.190	0.078	0.043	0.029	0.077	0.026	0.025	0.074	0.026
P-value													
diet		0.426	0.0005	0.024	0.836	0.036	0.020	0.531	0.199	0.014	0.981	0.531	0.012
SE		0.002	<0.0001	0.0004	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	0.122	<0.0001	<0.0001
Interaction		0.262	<0.0001	0.019	0.568	0.0059	<0.0001	0.110	0.008	0.0007	0.296	0.413	0.0015

[a-d] Values with different superscripts in the same column are significantly different ($P < 0.05$) by Duncan's multiple range test (n = 8). Chicks were inoculated with 10^8 cfu *Salmonella* Enteritidis or PBS at 3 d of age. Dietary prebiotics were administered, starting on the day of hatch (The same as below).

Table 6-3 (continued)

Diet	SE	*TRAF6*			*NF-κB*		
		1DPI	7DPI	14DPI	1DPI	7DPI	14DPI
NC	-	0.998	0.972	1.024^{b}	1.008	0.98	0.983^{c}
	+	1.193	1.335	1.652^{a}	1.339	1.214	1.306^{a}
FOS	-	1.01	0.989	1.041^{b}	0.972	1.031	1.029^{c}
	+	1.101	1.192	1.114^{b}	1.258	1.097	1.150^{b}
SEM		0.027	0.040	0.060	0.030	0.026	0.028
P-value							
diet		0.400	0.262	0.0006	0.149	0.4356	0.039
SE		0.006	<0.0001	<0.0001	<0.0001	0.0015	<0.0001
Interaction		0.272	0.156	0.0003	0.576	0.052	0.0006

Table 6-4. Effects of FOS on expression of inflammatory genes involved in MyD88-dependent signaling in cecal tonsils.

Diet	SE	*IL-6*			*TNF-α*			*TLR4*			*MyD88*		
		1DPI	7DPI	14DPI	1DPI	7DPI	14DPI	1DPI	7DPI	14DPI	1DPI	7DPI	14DPI
NC	-	1.016	1.014	0.998^{c}	1.001	0.993^{c}	0.973^{c}	1.026	1.017^{c}	1.007^{c}	0.992	1.003^{c}	0.989^{c}
	+	0.997	1.684	1.412^{a}	1.151	1.744^{a}	1.909^{a}	1.211	1.525^{a}	1.297^{a}	1.140	1.510^{a}	1.200^{a}
FOS	-	1.111	1.041	1.202^{b}	1.078	1.216^{b}	1.216^{b}	1.086	1.046^{c}	1.143^{b}	1.030	1.021^{c}	1.056^{c}
	+	1.12	1.694	1.265^{b}	1.227	1.274^{b}	1.308^{b}	1.137	1.251^{b}	1.195^{b}	1.177	1.276^{b}	1.121^{b}
SEM		0.025	0.074	0.032	0.030	0.058	0.073	0.031	0.045	0.034	0.033	0.045	0.020
P-value													
diet		0.0335	0.762	0.0026	0.156	0.002	<0.0001	0.907	0.0003	0.006	0.663	0.0015	0.0007
SE		0.908	<0.0001	<0.0001	0.0094	<0.0001	<0.0001	0.058	<0.0001	<0.0001	0.467	<0.0001	<0.0001
Interaction		0.781	0.887	<0.0001	0.995	<0.0001	<0.0001	0.27	<0.0001	0.0001	0.910	0.0003	0.0001

Table 6-4 (continued)

Diet	SE	*TRAF6*			*NF-κB*		
		1DPI	7DPI	14DPI	1DPI	7DPI	14DPI
NC	-	1.008	1.011	1.003^{d}	0.996	1.019^{b}	1.003^{c}
	+	1.222	1.422	1.422^{a}	2.887	1.344^{a}	1.274^{a}
FOS	-	1.05	0.964	1.0925^{c}	1.054	1.022^{b}	1.028^{c}
	+	1.137	1.215	1.270^{b}	2.591	1.024^{b}	1.118^{b}
SEM		0.039	0.043	0.036	0.185	0.036	0.024
P-value							
diet		0.775	0.0068	0.285	0.206	0.0019	0.006
SE		0.055	<0.0001	<0.0001	<0.0001	0.0023	<0.0001
Interaction		0.407	0.072	0.004	0.065	0.0021	0.0004

3.3 Effects of FOS on expression of inflammatory genes in chicken macrophages

FOS at doses of 100 and 200 µg/mL significantly increased the relative expression of *TNF-α* ($P < 0.05$) and *IL-6* ($P < 0.05$) in chicken HD11 cells when compared with untreated cells (Fig. 6-1A), although the increase was much lower than that of in response to LPS. As shown in Figure 1B, pretreatment of HD11 cells with 200 µg/mL FOS significantly reduced the relative expression of *TNF-α* ($P < 0.05$) and *IL-6* ($P < 0.01$) at 6, 12 and 24 h after challenge with LPS when compared with no exposure to FOS. Similarly, lower concentrations of FOS also decreased the abundance of *TNF-α* at 12 h after LPS ($P < 0.01$), and 100µg/mL FOS reduced the expression of *IL-6* at 6, 12 and 24 h ($P < 0.05$) (Fig. 6-1B).

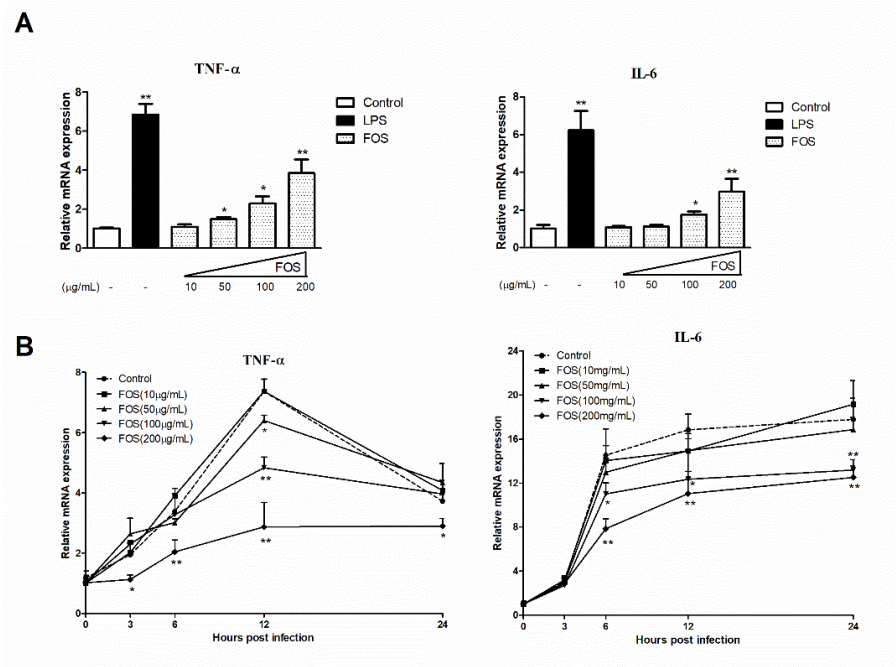

Figure 6-1. Effects of FOS on modulating inflammatory response of chicken macrophages. (A) Relative abundance of *TNF-α* and *IL-6* transcripts in untreated HD11 (control) cells or cells treated for 12 h with FOS (10, 50, 100, 200 µg/mL) or LPS (10 µg/mL); (B) Fold-change of *TNF-α* and *IL-6* transcripts in HD11 cells pretreated for 6 h with FOS (0-200 µg/mL) and challenged with LPS for different durations. The data are means ± SEM (n = 3). *$P < 0.05$ and **$P < 0.01$ versus control.

4. Discussion

Prebiotics exert beneficial effects on the immune system both indirectly, by promoting the growth of probiotic bacteria and increasing IgA secretion and production of SCFAs, and directly, by competitively inhibiting pathogen adherence and signaling (Forchielli and Walker, 2005; Vogt et al., 2015). So far, just a few studies have focused on the possible direct actions of prebiotics on the intestinal mucosa in rats (Zenhom et al., 2011; de Kivit et al., 2011; Capitán-Cañadas et al. 2014). Although the immune system of birds, which is different from that of mammals, is well characterized, the direct regulatory function of FOS in the innate immune defense against *Salmonella* in young infected chickens is still unknown. In the present study with young chickens, dietary addition of FOS was found to attenuate the expression of proinflammatory cytokine genes and inflammatory-related genes involved in MyD88-dependent signaling following *Salmonella* infection. Interestingly, FOS had a direct effect by reducing the expression of inflammatory factors in chicken cells. These findings suggest that FOS / inulin may also exert direct immunostimulatory effects in chickens.

In response to systemic infection with *Salmonella*, pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α), are produced by the innate and adaptive immune system (Chappell et al. 2009; Setta et al. 2012; Tohidi et al. 2018). Toll like receptor 4 (TLR 4) plays an essential role following *Salmonella* challenge and invokes the defense mechanism of the host organism that leads to synthesis of cytokines and related mediator molecules causing activation of intricate intracellular signaling pathways (Akira and Takeda, 2004; Takeda et al., 2003). In the present study, an upregulation in relative expression of inflammatory genes (*IL-6* and *TNF-α*) in the spleen and cecal tonsil was observed after *Salmonella* challenge, similar to previous findings (Chappell et al., 2009; Ciraci et al., 2010; Babu et al. 2012; Li et al., 2010). The effect of FOS in modulating LPS-provoked expression of proinflammatory cytokine was also investigated in vitro. Interestingly, the expression of *IL-6* and *TNF-α* gene in HD11 cells increased in response to FOS, in a concentration-dependent manner. This direct effect, although much smaller than that caused by LPS, would be beneficial in vivo for stimulating the host to produce a protective immune response. These results are consistent with findings that FOS/inulin addition upregulated the expression of proinflammatory cytokines in chicken ileum (Shang et al., 2015) and increased IL-6 and TNF-α secretion by splenocytes of WT and TLR4$^{-/-}$ mice (Capitán-Cañadas et al. 2014). It is suggested that FOS has a direct immunostimulatory effect by activating the host intestinal innate immune response.

Evidence suggests that prebiotics may also be effective for the prevention of inflammatory disorders, including sepsis (Manhart et al., 2003; Kovacs-Nolan et al.,

2013). The exaggerated inflammatory response typically leads to sustained systemic inflammation, which contributes to failure to clear pathogens and causes target tissue damage by causing defective innate and adaptive immune responses (Cohen, 2002; Li et al., 2017). IL-6 and TNF-α are also key mediators in the pathogenesis of sepsis and are rapidly induced after *Salmonella* / LPS administration (Meyer et al., 1995). In the present study, the transcript abundance of these inflammatory genes in spleen and cecal tonsils was reduced at 7 and 14 dpi in chicks fed FOS and infected with SE. In addition, the expression of inflammatory related genes involved in MyD88-dependent signaling (*TLR4*, *MyD88*, *TRAF6* and *NF-κB*) was also decreased at 14 dpi. There is an obvious time dependent effect of dietary FOS on reducing the expression of inflammatory factors and inflammatory related genes after SE infection. Potential direct effects of FOS in modulating expression of proinflammatory cytokines were examined in vitro using HD11 cells and challenge with LPS. Transcripts of both *IL-6* and *TNF-α* were decreased in chicken macrophages exposed to LPS after pretreatment with increasing concentrations of FOS; similar results have been obtained in rat primary monocytes (Capitán-Cañadas et al. 2014) and chicken macrophage (Babu et al., 2012). Recent studies showed that non-digestible oligosaccharides (FOS or inulin) are TLR4 ligands and directly modulate proinflammatory cytokine production via activation of TLR4-MyD88-NF-κB signaling in rat intestinal epithelial cells (Ortega-González et al., 2014) and in rat monocytes (Capitán-Cañadas et al. 2014). In addition, Kovacs-Nolan et al. (2013) reported that β-1,4-Mannobiose (MNB) modulates intestinal and systemic immune responses in healthy and endotoxemic mice and prevents LPS-induced immune suppression, as well as directly stimulating innate immune mechanisms in vitro as a TLR4 agonist. These results suggested that prebiotics may have a direct immunomodulation effect on TLR4 signaling to regulate the proinflammatory cytokine production in chicken.

Orally administered prebiotics are noninflammatory in themselves but are beneficial in experimental intestinal inflammation (Damaskos et al., 2008). Smaller polysaccharides (FOS, inulin and MNB) are considered to act as TLR4 agonists in rat intestinal cells and immune cells (Ortega-González et al., 2014; Kovacs-Nolan et al., 2013; Capitán-Cañadas et al. 2014). The effect of LPS was largely inhibited after TLR4 or MyD88 knockdown and was completely blunted in *TLR4* KO mice, and these effects were generally similar to those obtained with prebiotics. Prior exposure of immune cells to TLR2/4 agonists, however, can lead to their desensitization to a subsequent challenge with LPS, referred to as endotoxin tolerance (Dobrovolskaia et al., 2003), which prevents an excessive inflammatory response. The SCFA produced by intestinal fermentation of FOS in vivo also contribute by reducing inflammatory factors (Calik et al., 2016; He et al., 2017). Although the present study preliminarily demonstrated that FOS modulates the expression of inflammatory

factors involved in TLR4-MyD88-NF-κB signaling, further experiments are needed to evaluate the effect of FOS as an alternative TLR4 agonist in chicken intestinal cells and *TLR4* KO birds.

5. Conclusion

Taken together, these findings provide novel information that FOS may reduce the pro-inflammatory cytokines via TLR4-MyD88-dependent signaling during the early stages after *Salmonella* infection. However, further research of FOS direct immunomodulatory on TLR4 signaling is warranted.

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7

General Discussion and perspectives

Chickens are a good research model for *Salmonella* infection. As a foodborne disease, *Salmonella* Enteritidis causes huge economic losses to the poultry industry, and seriously threatens human public health (Calenge and Beaumont, 2012; Kogut and Arsenault, 2017). Poultry are considered to be important sources and carriers of the disease. Although *Salmonella* contamination can be significantly reduced using control measures in poultry, there was still a considerable increase in reported *Salmonella* cases in the EU (EFSA and ECDC, 2016) and UK (Inns et al., 2015). *Salmonella* Enteritidis also tends to be highly resistant to multiple antimicrobials, such as sulfamethoxazole-trimethoprim and nalidixic acid, which has the potential to complicate treatment of animal and human disease (DuPont and Steele, 1987; Goldman, 2004; Kuang et al., 2015). Therefore, to reduce economic losses in poultry production and to protect animal and human health, it is critical to understand better the host immune response to *Salmonella* infection in chickens.

Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease (Wang et al., 2009). In recent years, RNA sequencing (RNA-seq) has rapidly emerged as the major quantitative transcriptome profiling system (Mutz et al., 2013). Until now, mRNA and miRNA transcriptomic response to bacterial infection has been assessed utilizing RNA-seq to better understand the host response to *Salmonella* (Wu et al., 2017; Li et al., 2017), avian pathogenic *Escherichia coli* (Sandford et al., 2011, 2012; Nie et al., 2012; Sandford et al., 2011; Sun et al., 2015a, 2016a; Jia et al., 2017) and extraintestinal pathogenic *Escherichia coli* (Sun et al., 2015b, 2016b) in chickens.

In the present study, cohorts of *S.* Enteritidis challenged-susceptible (S), challenged-resistant (R) and non-challenged (C) chicks were used for transcriptome screening by RNA-seq. *Salmonella* resistance/susceptibility-related miRNAs, genes and signaling pathways were identified using the miRNA and mRNA transcriptome analyses. In addition, the effect of FOS on the innate immune resistance to *Salmonella* infection was also evaluated. These findings help facilitate understanding of host immune response to *Salmonella* infection in birds, provide new approaches to develop strategies for SE prevention and treatment, and may permit enhancing innate resistance by genetic selection. Because of logistical constraints, a complete understanding of these mechanisms is not yet complete and additional studies will be required. The present chapter provides a discussion of the contributions and conclusions of the study along with its limitations and future prospects.

1. miRNAs and genes involved in the host response to *Salmonella* infection

This study used a novel design that differed from those used previously where comparison was limited to *S.* Enteritidis challenged versus non-challenged birds (Li et al., 2010; Luan et al., 2012; Matulova et al., 2012, 2013; Wu et al., 2017). Indeed, it is complex and not easy to define the resistance and susceptible chickens following the *Salmonella* challenge. Recently based on degrees of clinical symptoms and pathological changes, previous research had constructed the susceptibility and

resistance model for APEC and ExPEC challenge in chickes (Jia et al., 2016; Nie et al., 2012; Sandford et al., 2011; Sun et al., 2015, 2016). In addition, the bacteria burden in tissues and blood is a commonly used method to assess disease resistance (Deng et al., 2008; Gou et al., 2012; Li et al., 2010). The unique design used here (Chapter 3 and 4) incorporated both the degree of clinical symptoms (diarrhea, drooping wings and dying), pathological changes (hepatomegaly, intestinal wall thickness and hemorrhage) and bacterial load of SE in blood to distinguish two pathology extremes in infected birds: resistant (R, SE challenged-slight clinical symptoms and $< 10^5$ cfu / 10 μL), and susceptible (S, SE challenged-severe clinical symptoms and $> 10^7$ cfu / 10 μL). In fact, it is only a relative definition for susceptibility and resistance, which used to distinguish the difference of immune response on SE infection among individual chickens.

To find the maximal different phenotypes in resistance and susceptible, we performed clinical pathological phenotypes and bacterial load at six time-points after infection. Groups of 30 SE-challenged chicks and controls were initially screened at 0.5, 1, 2, 4, 6 and 8-days post infection (dpi) at 3 d of age to identify when maximal differences (clinical symptoms and bacterial burden) occurred among the 3 groups; 1-dpi was chosen as likely to best distinguish potential differences in splenic mRNA and miRNA expression. Only at 1 dpi, the bacterial burden in blood of S chicks exceeded that in R chicks (Fig. 7-1, $P < 0.01$). At this time-point, blood bacteria and clinical pathological symptoms were closely correlated. In particular, individuals with severe clinical symptoms but without high blood counts were also excluded from this study. A solid foundation was provided for exposing mechanistic differences between the 3 groups by RNA analyses.

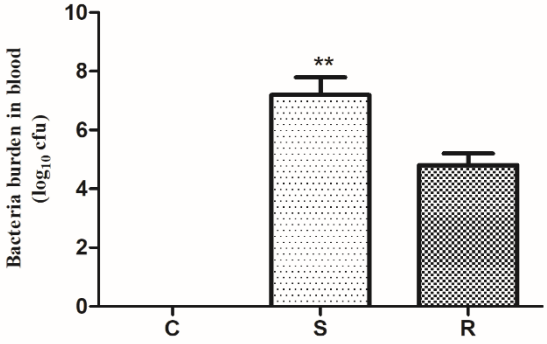

Figure 7-1. Bacterial burden in peripheral blood among C, R and S chicks at 1-day post infection. Data are presented as $\log_{10}$ of the bacterial genome copy number per 10μL of peripheral blood. Serovar-specific qPCR was used to quantify the S. enteritidis in blood, as described in Li et al. 2017. Six chicks in each group were selected. No *S.* Enteritidis was detected in the Controls. Each bar represents Mean ± SEM. ** indicate significant differences ($P < 0.01$) between S and R group.

In this preliminary experiment, the correlation between splenic bacterial burden and that in blood was also determined. As shown in Fig. 7-2, the number of SE ($\log_{10}$

cfu) measured in blood and spleen were closely related (R^2 = 0.892, n = 30).

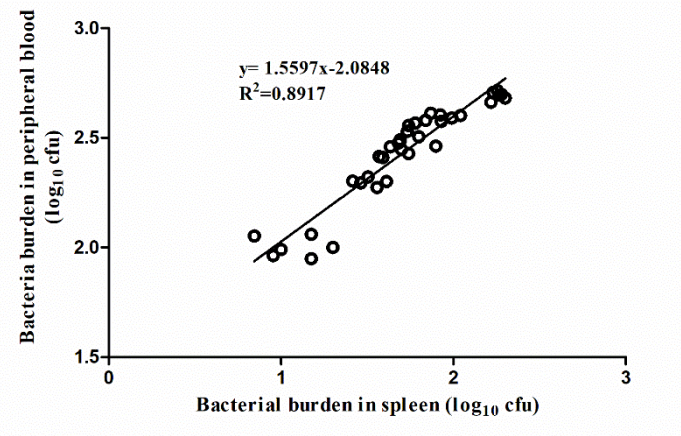

Figure 7-2. Analysis of the correlation between the amount of bacterial burden in peripheral blood and spleen tissue. Data are presented as the bacterial loads of five chickens on 0.5, 1, 2, 4, 6 and 8-days post infection. The X-axis represents the amount of bacteria in the spleen tissue and is expressed as $\log_{10}$ of the colony forming units per gram of tissue. The Y-axis represents the amount of bacteria in peripheral blood and is expressed as $\log_{10}$ of the bacterial genome copy number per 10μL of blood.

Different bacterial strains, virulence and dosage, and different clean grade animals will have a significant impact on the results of artificial challenge experiments. In present study, we purchased *Salmonella enterica* serovar Enteritidis, CMCC50041, from the China Institute of Veterinary Drugs Control (IVDC). It is a medium-low toxicity strain. Our previous studies have showed that the choice of 10^8 cfu of S. Enteritidis as the dosage to orally challenge is appropriate (Gou et al., 2012; Li et al., 2010, 2017). And the clinical symptoms of chicks with SE infection are mostly obvious within 1 week and deaths peak on the second day after infection. However, other SE challenge doses have also been reported in SPF chickens, such as 10^4 cfu of *S. enterica* serovar Enteritidis 1009 (Duchet-Suchaux et al., 1995; Sadeyen et al., 2004). This difference is normal and requires a preliminary experiment to assess the reasonable dose of the challenge. As mentioned above, we achieved the consistency of clinical pathological parameters and bacterial load by dynamically monitoring the changes at different days post infection.

Increasing evidence supports the avian spleen playing a greater role in immune function than in mammalian species, and in being responsible for an immediate immune reaction after recognizing pathogens by filtering antigens from the blood (Smith and Hunt, 2004; Li et al., 2017). The splenic transcriptome, thus, has been widely used in modeling disease infection in chickens, such as avian pathogenic *Escherichia coli* (APEC) (Nie et al., 2012; Sandford et al., 2011) and viruses (Haq et al., 2010; Smith et al., 2011; Wang et al., 2006).

The great advantage of the transcriptome is that large-scale screening and identification of candidate genes is practical using RNA sequencing. In the context of the present study, further functional enrichment analysis of these genes can help better understand the key points of immune resistance to *Salmonella* infection. As shown in Chapter 3, a total of 934 significant DEGs were identified in comparisons among the C, R and S birds. As expected, 265 DEGs were also found in R versus S chicks. Although several identified pathways and GO terms were enriched in previous splenic transcriptome studies (Matulova et al., 2012; Zhou and Lamont, 2007), some were uniquely identified here. For instance, there was cross-talk among these pathways, including cytokine-cytokine receptor interaction and Jak-STAT, perhaps contributing to susceptibility to *Salmonella* infection. Importantly, TLR4 signaling was also significantly enriched among C, R and S birds. Interesting, and first reported here, the DEG involved in the Forkhead box O (FoxO) signaling pathway, especially *FoxO3*, were identified as potential markers for host resistance to SE infection. These results add current understanding of the host immune response to *S.* Enteritidis in challenge and normal conditions, as well as exposing host differences with pathological severity of the disease.

MiRNAs are important regulators of the innate immune response induced by bacteria (Das et al., 2016; Eulalio et al., 2012; Maudet et al., 2014; Staedel and Darfeuille, 2013). Until now, there is strictly limited information about the function of miRNAs in the host response and resistance to *Salmonella* infection in chickens (Wu et al., 2017). As shown in Chapter 4, splenic miRNA profiles from chickens after *Salmonella* challenge have identified differential expression of several miRNAs linked to immune responses, including miR-155, miR-9, miR-30 which have been reported previously and several others, such as miR-101-3p and miR-130b-3p, which were first shown here to be associated with the immune response to infection with SE. miR-155 has been reported to play important roles in both innate and adaptive immunity in mammals. Its expression is up-regulated after activation of the innate response in murine macrophages by lipopolysaccharide, CpG and poly (I:C) and it can down-regulate these signaling pathways by targeting key signaling molecules (Elton et al., 2013; Li and Shi, 2013; Maudet et al., 2014; Olivieri et al., 2013). In Chapter 4, gga-miR-155 was shown to be significantly induced by SE infection, which was consistent with the above mammalian studies. Interestingly, the expression of gga-miR-155 was significantly higher in the S chickens compared with R birds. Several previous studies have demonstrated that *LRRC59* and *IRF4* are involved in regulation of TLRs signaling (Tatematsu et al., 2015; Honma et al., 2005; Negishi et al., 2005). Based on the above findings and other studies, it is reasonable to propose that gga-miR-155 and gga-miR-101-3p contribute to SE-induced pathogenesis and are involved in TLR signaling pathways through directly down-regulating *LRRC59* and up-regulating *IRF4* genes, respectively. In addition, miRNA-target genes were also enriched in innate immune signaling in R chicks. These investigations in Chapter 4 indicate that miRNAs in spleen play a major role in the SE infection process. In the present study the chicken macrophage cells HD11 were used to verify the function of miR-155 and miR-101. Similar research was also found by Jia et al. (2016). Overexpression of APEC-associated miRNA, gga-miR-429, which identified through the spleen transcriptome was performed on HD11 cells

(Jia et al., 2016). As the B and T lymphocytes are the mainly cells in the chicken spleen, further in-depth verification of miR-155 and miR-101 on lymphocytes is warranted.

It seems that susceptible chickens are more likely to exhibit larger gene changes and are more sensitive to *Salmonella* infection. Similar results were found in APEC infections in chicken primary lymphoid tissues (Sun et al., 2015, 2016). These results suggest that excessive production of inflammatory factors via the immune response in susceptible chickens' cause damage to the target tissues during *Salmonella* invasion, which in turn leads to endotoxic shock or sepsis-related deaths (Cohen, 2002; Kurtz et al. 2017; Netea et al. 2017). Further exploration of the mechanism underlying susceptibility to *Salmonella* challenge is warranted.

Taken together, these results in Chapter 3 and 4, indicate that multiple signaling pathways cross-talk when regulating the host immune resistance response to *Salmonella* infection. Key breakthroughs from the present research such as exposing FoxO and TLR4 signaling pathways, are worthy of additional research. Meanwhile the miRNAs, identified here, miR-155 and miR-101 play a precise role in TLR signaling under *Salmonella* infection in birds. These findings will facilitate the understanding of resistance and susceptibility to SE infection in the earliest phases of the host immune response and provide new avenues for developing strategies for SE prevention and treatment. Based on the above considerations, focus is now placed on the immunomodulatory effect of fructooligosaccharides (FOS) on the expression of pro-inflammatory factors via the TLR4 signaling pathway.

2. FOS modulates the host immune response to *Salmonella* infection

Prebiotics exert beneficial effects on the immune system both indirectly, by promoting the growth of probiotic bacteria and increasing IgA secretion and production of SCFAs, and directly, by competitively inhibiting pathogen adherence and signaling (Forchielli and Walker, 2005; Telg and Caldwell, 2009; Shang et al., 2015). SCFAs and *Bifidobacteria* are considered to be involved in the indirect mechanism by which FOS is immunomodulatory. The gut microbiota synthesizes a broad spectrum of hydrolases (Backhed et al., 2005) that digest complex dietary carbohydrates to monosaccharides and SCFAs such as acetate, propionate, and butyrate. Propionate and acetate are ligands for two G protein-coupled receptors (GPCRs), Gpr41 and Gpr43, mainly expressed by intestinal epithelial cells (Brown et al., 2005; Le Poul et al., 2003; Tilg and Kaser, 2011). Previous studies suggest that an immunoregulatory effect for SCFA-mediated G protein-coupled receptor (GPR) 43 signaling in mice (Maslowski et al., 2009; Meijer et al., 2010). Although in general, the production of *Bifidobacteria* by prebiotic fermentation has a bifidogenic effect (Kim et al., 2011, Peinado et al., 2013), there are inconsistencies in the prebiotics throughout the literature (Zhang et al., 2003; Jiang et al., 2006; Biggs et al., 2007). Direct mechanisms of immunomodulation by FOS are thought to entail ligand of pathogen recognition receptors (PRRs) on the surface of epithelial cells and immune cells (Vogt et al., 2015). Recent studies in rat intestinal epithelial cells (Ortega-González et al., 2014) and in rat monocytes (Capitán-Cañadas et al. 2014) showed

that nondigestible oligosaccharides (FOS or inulin) are TLR4 ligands and directly modulate production of pro-inflammatory cytokines via activation of TLR4-MyD88-NF-κB signaling. The possible mechanism is that prior exposure of immune cells to TLR2/4 agonists leads to their desensitization to a subsequent challenge with LPS, referred to as endotoxin tolerance (Dobrovolskaia et al., 2003), which prevents an excessive inflammatory response.

The present study mainly attempted to evaluate the possible therapeutic effect of dietary FOS supplementation on *Salmonella* infection, thus different concentrations levels of FOS-treated chicks were orally challenged with or without SE on d 3. This time of challenge is consistent with the earlier experiment (Chapter 3 and 4).

The aim of this Chapter was to investigate whether FOS addition alters the expression of inflammatory genes involved in MyD88-dependent signaling following *Salmonella* infection. In order to achieve this goal, it was necessary to screen the optimum dietary FOS providing protection against *Salmonella* infection. In Chapter 5, lower concentrations of FOS supplementation not only reduced the bacteria burden in liver and cecum, but also decreased the serum levels of interleukin-1β, as well as the expression of *TNF-α* and *IL-6* in spleen and cecal tonsils. As mentioned above, the birds fed dietary FOS (1%) had better immune protection than FOS (3%). The possible reason is that high doses of FOS is excessive fermented in the cecum leading to intestinal damage. The main function of FOS in the body is to produce SCFAs through fermentation. And this is beneficial in a certain amount, especially for chicks whose intestines are not yet well developed. Song et al. (2018) reported that inulin (0.5%-1%) significantly decreased ($P < 0.05$) the gene expression of *NF-κB*, *TNF-α*, *IL-6*, *iNOS*, and increased the mucin 2 and claudin-1 gene expression in no-challenged chickens. However, high inulin supplementation (2%) increased the expression of inflammation related genes and decreased the mucin 2 gene expression. These suggest that high concentrations of FOS may break the intestinal barrier and cause inflammatory reaction.

Controlling and reducing *Salmonella* colonization is an important measure to evaluate the effect of FOS on against *Salmonella* infection. A similar study found that FOS-treated mice showed lower mortality and incidences of aberrant crypt foci than did control mice when exposed to dimethylhydrazine or *Salmonella* Typhimurium (Buddington et al. 2002). In addition, FOS-inulin alleviated mucosal damage through decreasing gene expression of *TNF-α*, *IL-1α*, *IL-1β*, and *iNOS* in rat mucosal tissue (Ma et al. 2018; Wilson and Whelan, 2017), and increased phagocytic activity and secretory capacity against LPS or SE challenge of immune cells (Babu et al. 2012; Capitán-Cañadas et al. 2014; Neyrinck et al. 2004). The results presently obtained suggest beneficial effects of adding FOS, probably at 1%, to the diet of young chicks on protection against SE infection.

Also investigated *in vivo* were the effects of dietary FOS addition on the expression of inflammatory genes and TLR4 signaling genes (Chapter 5). An up-regulation in relative expression of inflammatory genes (*IL-6* and *TNF-α*) in the spleen and cecal tonsil was observed after *Salmonella* challenge, similar to previous findings (Chappell et al., 2009; Ciraci et al., 2010; Babu et al. 2012; Li et al., 2010). In addition, the transcript abundance of these inflammatory genes and those of TLR4-

MyD88-dependent signaling (*TLR4*, *MyD88*, *TRAF6* and *NF-κB*) were also reduced in spleen and cecal tonsils. It is suggested that maybe the effect of dietary FOS on reducing the expression of inflammatory factors after *Salmonella* infection was mediated by TLR4 signaling. However, as mentioned earlier, the SCFA produced by intestinal fermentation of FOS *in vivo* also contribute by reducing inflammatory factors (Calik et al., 2016; Weitkunat et al., 2016; Koh et al., 2016). SCFA can reduce the expression of host inflammatory factors by decreasing the number of pathogenic bacteria. Thus, it seems likely that both mechanisms contribute to the FOS immunomodulatory effect *in vivo* during *Salmonella* infection. Potential direct effects of FOS in modulating expression of pro-inflammatory cytokines were examined *in vitro*. Transcripts of both *IL-6* and *TNF-α* were decreased in chicken macrophages provoked by LPS after pretreatment with increasing concentrations of FOS; similar results have been obtained in rat primary monocytes (Capitán-Cañadas et al. 2014) and chicken macrophages (Babu et al., 2012).

Taken together, these findings provide novel information that FOS may reduce the production of pro-inflammatory cytokines through TLR4-MyD88-dependent signaling during the early stages after *Salmonella* infection. Further analysis of direct immunomodulatory actions of FOS on TLR4 signaling needs to be performed *in vitro*.

3. Limitations and prospects of future research

Although the research described here provides novel insights on *Salmonella* resistance-associated miRNAs, genes and signaling pathways, as well as the immunomodulatory function of FOS, some specific limitations remain. The following discussion analyzes the limitations and forecasts the future direction of this line of investigation.

(i) **The number of samples sequenced.** Although each group of 3 individuals met the sample number typically required for RNA sequencing, more biological replication would improve the quality and reliability of detecting differential expression using RNA-Seq (Robles et al., 2012). In future studies of disease models, the influence of individual differences on the experimental results can be further reduced by increasing biological repetition.

(ii) **Insufficient data mining and its effective use.** Compared to traditional methods (real-time quantification PCR and microchips, etc.), large-scale screening of candidate genes, including transcripts of very low abundance, is possible using RNA-Seq. Global gene expression platforms allow for the discovery of novel genes involved in the immune response, as a generic trait assay, giving greater insight into pathways and signal cascades that change during infection. Given the huge amounts of data able to be generated, there are many interesting follow-up studies that can emerge. In the present effort, focus was placed on aspects of innate immunity. In fact, other directions, such as closer examination of the role of the Foxo signaling pathway in the host immune response to *Salmonella* infection, would appear to be promising. In addition, further functional verification of the likely roles of miRNAs, such as miR-155 exposed here is needed, using chickens or chicken embryos. Functional analysis

of others of the 32 DE miRNAs identified here is also worthy of attention. For example, the newly discovered miR-1306, confirmed here, is known to regulate TLR4 signaling by its target, the Tollip transcript. In-depth data mining and analysis is necessary, therefore, and likely to be worthwhile.

(iii) **Effects of FOS addition *in vivo*.** The main focus of the present experiment was the effect of FOS on the expression of genes involved in TLR4-MyD88 signaling and pro-inflammatory cytokines. The colonization of *Salmonella* and expression of inflammation-related genes in peripheral immune organs were first measured in the feeding study, but this was quite preliminary in nature and little is known for chickens in vivo. Many factors come into play and possibly interfere in the intake and the digestive processeses; it is likely that dietary FOS has multiple actions in animal models. More phenotypic indicators deserve attention, such as fermentation production of SCFA including butyrate, although previous studies indicate that some indicators will be inconsistent (Zhang et al., 2003, Jiang et al., 2006, Biggs et al., 2007). Given the complexity of investigating prebiotics in vivo, the causative mechanism(s) of their regulating immune function will be difficult to discern unless a *TLR4*-KO model becomes available, presently difficult to achieve in birds. It seems that systematic consideration of interactions between microorganisms and the host immune system will be the focus of immediately future research. In addition, the developing of new high-throughput technologies, such as metagenomics and metabolomics, will help add understanding of the immunomodulatory mechanisms of probiotics *in vivo*.

(iv) **Effects of FOS exposure *in vitro*.** There is limited knowledge of the effects of FOS in chicken cells. A contributing reason is that many important antibodies and ligands, such as chicken's TLR4, MyD88, and NF-κB, have not been commercialized as in the case of mouse and human. Future studies will focus on the interaction between FOS and chicken TLR4 ligands. Assessment of direct immunoregulation of FOS will be aided by constructing *TLR4*-KO chicken cells, such as intestinal epithelial cells, heterophils and macrophages.

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Appendix – publications

1. **Peng Li**, Wenlei Fan, Qinghe Li, Jie Wang, Ranran Liu, Nadia Everaert, Jie Liu, Yonghong Zhang, Maiqing Zheng, Huanxian Cui, Guiping Zhao and Jie Wen. **(2017)**. Splenic microRNA expression profiles and integration analyses involved in host responses to Salmonella enteritidis infection in chickens. *Front. Cell. Infect. Microbiol* 7:377.
2. **Peng Li**, Wenlei Fan, Nadia Everaert, Ranran Liu, Qinghe Li, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. **(2018)**. Messenger RNA sequencing and pathway analysis provide novel insights into the susceptibility to Salmonella enteritidis infection in chickens. *Front. Genet*. 9:256.
3. **Peng Li**, Xingwang Zhao, Huihua Wang, Zhongyong Gou, Ranran Liu, Nadia Everaert, Guiping Zhao, Jie Wen. **(2017)**. Allelic variation in TLR4 is linked to resistance to *Salmonella* enteritidis infection in chickens. *Poult Sci* 96, 2040-2048.
4. Lei Zhang* & **Peng Li***, Ranran Liu, Maiqing Zheng, Dan Wu, et al. **(2015)**. The identification of loci for immune traits in chickens using a genome-wide association study. *PLoS ONE* 10(3): e0117269.
5. **Peng Li**, Weiwei Sun, Nadia Everaert, Ranran Liu, Qinghe Li, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. (2018). Screen the optimum concentration of dietary FOS for reducing the expression of pro-inflammatory factors during Salmonella infection in chicken. *Poult Sci.* (Submitted).
6. **Peng Li**, Weiwei Sun, Nadia Everaert, Ranran Liu, Qinghe Li, Maiqing Zheng, Huanxian Cui, Guiping Zhao, Jie Wen. Effects of FOS in modulating the expression of inflammatory genes involved in MyD88-dependent signaling following Salmonella infection in young chickens. (Prepare to submit)